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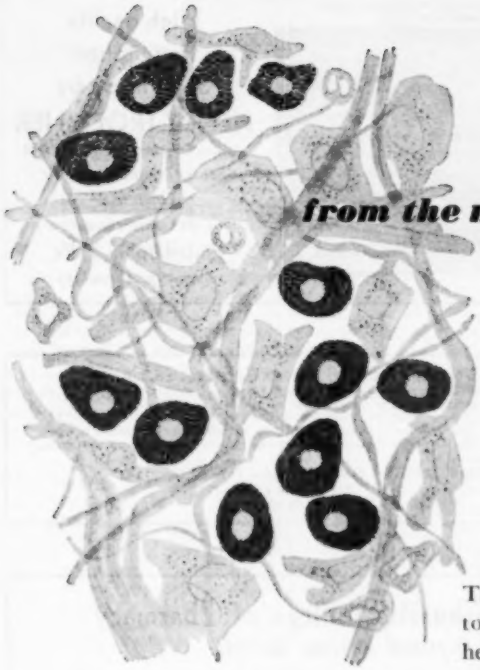

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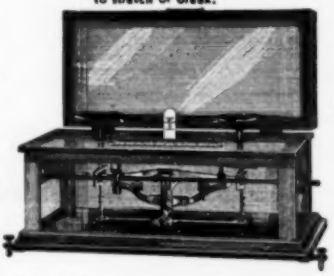

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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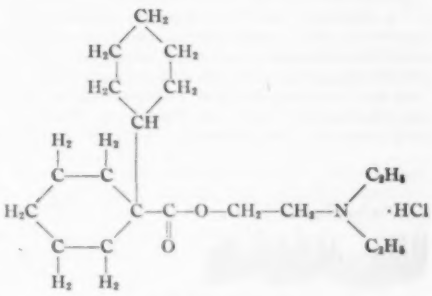
Pharmacological Studies on the Antispasmodic, β-Diethylaminoethyl 1-Cyclohexylcyclohexanecarboxylate Hydrochloride*

By BARBARA B. BROWN, CHARLES R. THOMPSON, GERTRUDE R. KLAHM, and HAROLD W. WERNER

A new compound, Bentyl hydrochloride (β-diethylaminoethyl 1-cyclohexylcyclohexanecarboxylate hydrochloride), has been demonstrated to possess parasympathetic depressant properties. It has a marked relaxing action *in vivo* on normal and barium-stimulated intestine, and it strongly antagonizes the intestinal effects of acetylcholine. The bulk of Bentyl administered to animals appears to be destroyed in the body, and the substance has low toxicity following single and repeated administration to animals.

THE SYNTHESIS of a large series of aminoesters of substituted alicyclic carboxylic acids has been reported by Tilford, *et al.* (1, 2). The *in vitro* antispasmodic activity of these compounds described in his report indicated that several compounds possessed high antiacetylcholine action.

One of these compounds, β-diethylaminoethyl 1-cyclohexylcyclohexanecarboxylate hydrochloride, has received extensive pharmacological evaluation and is proving valuable in clinical studies. It is a colorless and odorless white solid which is water-soluble to approximately 25 per cent. The compound has the name Bentyl hydrochloride[1] and has the following structure:



EXPERIMENTAL

Isolated Intestine.—The activity of Bentyl hydrochloride was determined on the isolated intestine according to the method of Magnus and was compared to the activity of other antispasmodic compounds. Two- to three-centimeter segments of rabbit jejunum were suspended in 100-cc. baths of

* Received December 9, 1949, from the Pharmacology Department, Research Laboratories, The Wm. S. Merrell Co., Cincinnati, Ohio.

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aerated Locke's solution maintained at 38 ± 0.5°. Minimal concentrations which relaxed the normal intestine and the spasms induced by acetylcholine chloride and barium chloride were determined.

The results in Table I indicate that Bentyl is less potent on a milligram basis than atropine and Pavatrine on the untreated intestinal segment. It is slightly more effective than papaverine against the direct muscular stimulation produced by barium. The milligram potency of Bentyl hydrochloride against the parasympathetic stimulation produced by acetylcholine is one-eighth that of atropine and considerably greater than that of all other substances tested.

TABLE I.—ACTIVITY OF SEVERAL ANTISPASMODICS ON THE ISOLATED RABBIT INTESTINE

Compound	Minimal Effective Concentration		
	Normal Intestine	Against Acetylcholine (1:1,000,000) Spasm	Against $BaCl_2$ (1:10,000) Spasm
Bentyl hydrochloride (β-diethylaminoethyl 1-cyclohexyl-cyclohexanecarboxylate·HCl)	1:100,000	1:10,000,000	1:200,000
Trasentine (β-diethylaminoethyl diphenylacetate·HCl)	1:50,000	1:1,000,000	1:200,000
Pavatrine (β-diethylaminoethyl fluorene-9-carboxylate·HCl)	1:2,500,000	1:1,000,000	1:500,000
Atropine sulfate	1:10,000,000	1:80,000,000	1:200,000
Papaverine·HCl	1:150,000	1:100,000	1:100,000

Acetylcholine Antagonism.—The parasympathetic depressant activity of Bentyl and atropine was compared by measuring their blocking action of the intestinal response to acetylcholine in cats (3). A total of nine cats was used for these experiments. The animals were anesthetized with 40 mg./Kg. of Amytal Sodium (amobarbital sodium) intravenously. Jejunal activity was recorded by means of an air tambour-balloon system, and carotid blood pressure was recorded simultaneously. Activity evaluations were made by finding the smallest intravenous dose of atropine sulfate and of Bentyl hydrochloride which blocked a standard intestinal response to acetylcholine. Standard responses to acetylcholine chloride were obtained by repeated intravenous injections of 0.01 mg./Kg. doses.

Results of a typical experiment are shown in Fig. 1. A dose of 2.5 mg./Kg. of Bentyl hydrochloride produced a transitory drop in blood pressure, a sharp fall of intestinal tonus, and almost completely inhibited intestinal contractions for twenty minutes. This dose also completely or partially blocked the intestinal response to acetylcholine for forty to sixty minutes. Smaller doses, 0.5 or 1.0 mg./Kg. of Bentyl, were effective for shorter periods, and they had a marked direct depressant action on intestinal motility. The same type of action was produced by 0.005 mg./Kg. of atropine.

Barium Antagonism.—A number of experiments were conducted in anesthetized dogs employing barium chloride instead of acetylcholine to produce intestinal spasm. An intense intestinal spasm induced by the intravenous injection of 3 mg./Kg. of barium chloride was promptly and completely relaxed by the intravenous injection of 2.5 mg./Kg. of Bentyl hydrochloride. A subsequent barium injection given three minutes later usually had no intestinal stimulant action. Doses of 1 mg./Kg. of atropine sulfate failed to relieve the barium spasm and did not inhibit the action of a subsequent injection of barium.

Thiry-Vella Dogs.—The relaxing action of Bentyl on the untreated intestine of the unanesthetized animal was determined in four dogs. Thiry-Vella loops of the upper jejunum had been prepared and the dogs trained for approximately a month before drug studies were begun. Intestinal activity was recorded by means of a closed pressure system. Test compounds were injected intravenously after recording control periods of fifteen to twenty minutes of normal intestinal activity.

The tracing of a typical experiment is shown in Fig. 2. The record indicates that 2 mg./Kg. of Bentyl hydrochloride caused a decrease of intestinal tonus and a marked and prolonged depression of contractions. The effect persisted for approximately forty minutes. A dose of 1 mg./Kg. also produced marked intestinal depression but for a shorter period of time.

Comparative studies using Trasentine and Pavatrine indicated that Trasentine had a similar depressant action, whereas the action of Pavatrine

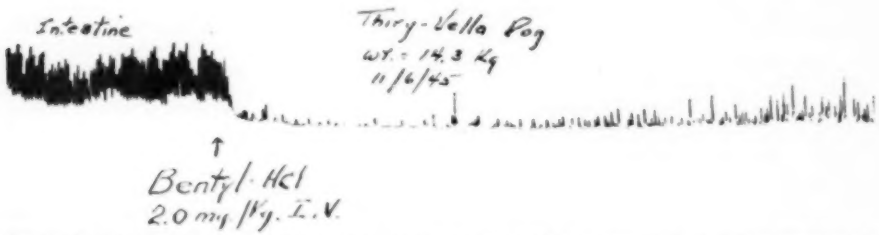

Fig. 1.—The intestinal action of 2.5 mg./Kg. of Bentyl hydrochloride intravenously in the anesthetized cat. Intestinal motility is markedly inhibited and the intestinal response to acetylcholine is blocked.

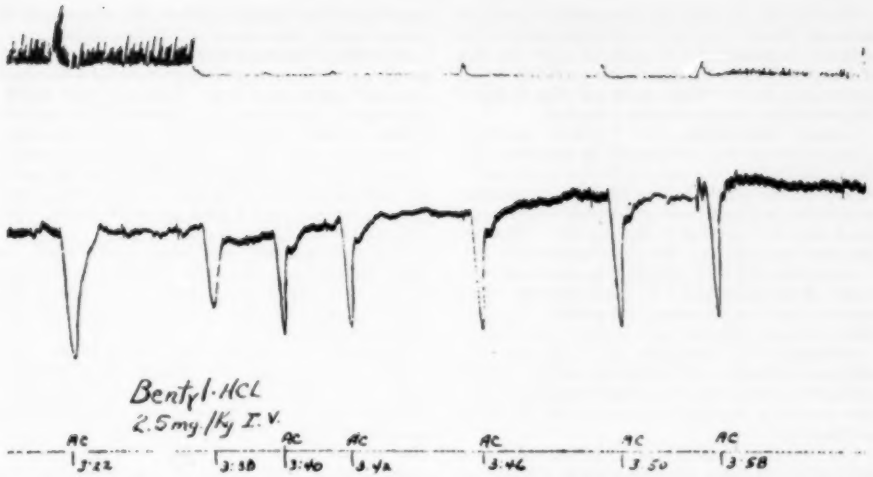

Fig. 2.—Relaxation of the intestine in an unanesthetized Thiry-Vella dog produced by 2 mg./Kg. of Bentyl hydrochloride intravenously.

was weaker and less consistent. This difference is of interest since Pavatrine is considerably more depressant than either Bentyl or Trasentine on the isolated rabbit intestine.

Activity Orally in Rats.—Further indication of the antispasmodic action of Bentyl in the unanesthetized animal was obtained following oral administration in rats. The method used was a modification of the procedure described by Macht and Barba-Gose (4). The drug was given orally, followed in thirty minutes by a charcoal meal (5% charcoal, 5% gum acacia in water) also administered orally. Twenty minutes later the rats were killed with ether, and the small intestine removed and measured. The distance the charcoal meal had traversed was measured and expressed as per cent of total length of the small intestine. The difference between this value for treated rats and for control rats forms a measure of the antispasmodic activity of compounds which have been demonstrated to have a depressant effect on both intact and isolated intestinal muscle.

Experimental results are given in Table II. The charcoal meal traversed approximately 63% of the small intestine in twenty minutes. A dose of 1 mg./Kg. of atropine sulfate decreased this distance to approximately 30%. A dose of 150 mg./Kg. of Bentyl hydrochloride caused a similar delay and a dose of 300 mg./Kg. markedly inhibited movement of the meal.

TABLE II.—EFFECTS OF ORAL ADMINISTRATION OF BENTYL HYDROCHLORIDE AND ATROPINE SULFATE ON THE PASSAGE OF A CHARCOAL MEAL IN RATS

Compound	Dose, Mg./Kg.	No. of Rats	Intestine Traversed, %
Controls	...	16	63.5 ± 1.9
Atropine sulfate	1	12	30.0 ± 1.97
Bentyl hydrochloride	50	8	49.8 ± 5.2
	150	16	42.5 ± 2.3
	300	8	15.2 ± 4.5

Comparative studies indicated that 300 mg./Kg. of Pavatrine hydrochloride was required to effect a 40% decrease and that doses of 300 mg./Kg. or less of Trasentine hydrochloride did not cause any delay in passage of the charcoal meal.

Isolated Ureters.—The activity of Bentyl was compared to that of other antispasmodics on isolated dog ureters. Recording was done by the method of Magnus employing Locke's solution as a bath. Spontaneous activity was obtained by adding urea in a 1:20 dilution of dog urine to make a 1:10 or 1:20 dilution.

Concentrations of the compounds in milligrams per 100 cc. of bath which inhibited ureteral contractions were 5 for Bentyl and Pavatrine, 10 for Trasentine, and 25 or more for atropine.

Mydriatic Action.—The procedure of Ing, *et al.* (5) was followed to evaluate mydriatic action. Five mice, weighing between 18 and 20 Gm. each, were employed for each dosage group. Pupil size was measured by means of an ocular micrometer and a microscope giving 20 magnifications. Injections of the drugs in 0.2-cc. volumes were made intraperitoneally, and pupil size was determined fifteen minutes after drug administration when maximal dilatation had occurred.

Maximal dilatation occurred in 80% or more of the pupils tested following the administration of 0.008 mg. of atropine sulfate. In comparison, doses of 4.0 mg. of the hydrochlorides of Bentyl, Trasentine or Pavatrine were required to produce maximal dilatation.

Salivary Secretion.—Antisialogogue action was determined in rabbits anesthetized with 1.1 Gm./Kg. of urethane administered orally. Salivary secretion was stimulated by the intramuscular administration of 5 mg./Kg. of pilocarpine nitrate. Effects of the test substances were evaluated by injecting them intramuscularly along with the pilocarpine. Six rabbits were used per dose level.

The average secretion for pilocarpine control animals was 69 and 71 cc. for a two-hour period in two separate experiments. A dose of 0.15 mg./Kg. of atropine sulfate decreased the secretion to approximately 20 cc. Doses of 48 mg./Kg. of Bentyl hydrochloride caused a similar reduction.

Corneal Anesthesia.—The topical anesthetic action of Bentyl was investigated by instilling aqueous solutions into the conjunctival sacs of rabbits for one-minute periods. The duration of anesthesia was determined by following the wink response to a small glass rod touched to the cornea. Three rabbits were used for each concentration studied.

A solution of 0.25% of Bentyl hydrochloride produced deep anesthesia of approximately thirty minutes, and a 0.5% solution produced deep anesthesia of approximately sixty minutes' duration.

Irritation.—The instillation of 0.25% and 0.5% solutions of Bentyl hydrochloride into the rabbit's conjunctival sac produced a slight temporary irritation consisting of mild redness and edema of the conjunctiva.

The subcutaneous injection of 1.0-cc. volumes in rats caused no tissue damage when a 1% solution was used, and a 2% solution caused only minimal necrosis in half of the animals injected.

Acute Toxicity.—The intravenous LD_{50} of Bentyl hydrochloride for rabbits is 35 ± 3.3 mg./Kg. Doses of one LD_{50} or higher caused convulsions lasting about two minutes followed by a depression of one to three hours in surviving animals. Deaths occurred within twenty minutes after injection.

The LD_{50} of Bentyl hydrochloride for mice following oral administration is 625 ± 21 mg./Kg. Convulsions appeared about thirty minutes after drug administration and were followed by a depression lasting three to five hours. The majority of deaths occurred within one hour.

It was observed incidentally that the intravenous administration of Bentyl hydrochloride was followed at times by hemoglobinuria. Further study demonstrated that Bentyl produced hemoglobinuria when given intravenously to rabbits in doses of 5 mg./Kg. or more and to rats in doses of 10 mg./Kg. or more.

Intravenous doses of 5 mg./Kg. to dogs and 20 mg./Kg. to mice were not followed by hemoglobinuria; and subcutaneous, intramuscular, and oral administrations did not lead to the appearance of hemoglobin in the urine in any of the species.

Repeated Oral Administration to Dogs.—Fifteen adult mongrel dogs of both sexes were fed a standard diet with regulated caloric intake, and were conditioned to the laboratory for a period of three months before starting the experiment. They were then divided into three dose groups of five animals each. Two groups were administered Bentyl hydrochloride in doses of 4 and 10 mg./Kg., respectively, three times a day, six days a week, for eight weeks; and the third group served as a control.

The antispasmodic was administered orally in hard gelatin capsules, and capsules containing sucrose were administered to the controls since this substance was employed as a diluent in capsules for the experimental groups.

Hematologic evaluations were made every other week for all animals. The hemoglobin determinations were made with a Fisher electrohemometer, and the hematocrit determinations were conducted according to the Wintrobe technique (6), except that minimal amounts of a 10% solution of potassium oxalate were added as an anticoagulant. Sedimentation rates were corrected for erythrocyte volume by the method of Wintrobe and Landsberg (7) employing a standard normal value derived from data obtained in this laboratory.

At the termination of drug administration all dogs were in excellent physical condition. All groups exhibited weight gain (see Table III).

Thirteen of the 15 dogs maintained a fairly constant increase in body weight. One control animal lost 0.5 Kg., and the weight of one dog in the 10 mg./Kg. dose group fluctuated considerably.

Bentyl hydrochloride did not cause blood dyscrasias (see Table IV). The erythrocyte counts, total and differential leucocyte counts, hemoglobin concentrations, hematocrits, icterus indices, and sedimentation rates of the experimental groups were normal; and the values did not differ significantly from those of the controls.

No gross pathologic changes were observed that could be attributed to drug administration. The intestinal mucosa of one control animal and one animal from each of the two drug groups was ulcerated. These animals both exhibited heavy infestations of intestinal parasites.

TABLE III.—INITIAL AND FINAL BODY WEIGHTS OF CONTROL AND EXPERIMENTAL ANIMALS ADMINISTERED REPEATED DOSES OF BENTYL HYDROCHLORIDE

Dose Group, Mg./Kg.	Animals	Body Weight—Initial—Mean Kg.	Body Weight—Initial—Range Kg.	Body Weight—Final—Mean Kg.	Body Weight—Final—Range Kg.
4, t.i.d.	Dog	8.0	5.5–11.7	8.9	5.7–11.9
10, t.i.d.		8.2	5.0–14.8	8.4	5.5–13.7
Control		8.0	4.0–15.2	8.7	3.9–16.4
4, t.i.d.	Monkey	...	5.7	...	6.5
		Gm.	Gm.	Gm.	Gm.
20	Rat	142	130–165	245	195–320
100		143	120–170	252	190–350
500		149	125–190	250	190–350
100*		144	110–170	231	180–290
Control	Rat, 24 weeks	136	110–160	250	190–350
10, b.i.d.	administration	123	112–140	259	194–330
Control		122	110–148	267	198–372

* Food withheld four hours before and after dosing.

The microscopic changes observed in tissue specimens from the aorta, heart, lung, liver, spleen, adrenal, kidneys, urinary bladder, stomach, and small and large intestines were minimal and were of types frequently observed in dogs. They occurred as frequently in the control animals as in those administered Bentyl and, therefore, were not related to drug administration.

Daily Oral Administration to Rats for Eight Weeks.—The effects of repeated stomach tube administration of Bentyl hydrochloride were evaluated in five groups of five male and five female rats each. One of the groups was administered water and served as a control. Three groups were administered Bentyl hydrochloride in single daily doses of 20, 100, and 500 mg./Kg. and were allowed food *ad libitum*. Food was withheld from the fifth group for four hours preceding and following daily doses of 100 mg./Kg. of the antispasmodic. All doses were administered in a volume of 5 cc./Kg., six days a week over a period of eight weeks. The animals were sacrificed after eight weeks of drug administration.

Three accidental deaths occurred during drug administration; two in the 500 mg./Kg. dose group, and one in the starved 100 mg./Kg. group. These deaths resulted from intubation of the lungs at the time of drug administration and were not related to chronic oral drug toxicity. All other experimental and control animals appeared normal throughout the experiment. The daily doses of Bentyl hydrochloride employed did not alter body weight (see Table III). Erythrocyte, total and differential leucocyte counts and hemoglobin concentrations remained in the normal range; and values for experimental groups did not differ significantly from those for the controls (see Table IV).

Daily oral doses of 20 mg./Kg. of Bentyl hydrochloride did not produce histologic tissue alterations. Animals administered daily oral doses of 100 and 500 mg./Kg. exhibited a mild, fatty, or hydropic degeneration of the parenchymatous cells of the liver. This condition was observed in the livers of approximately one-half of the animals of each of the two 100 mg./Kg. groups, all of the animals in the 500 mg./Kg. group, and in one control animal. The nuclei of the cells showing this change were normal. Other tissues which included those described in the experiment on dogs showed no significant histologic alterations.

Daily Oral Administration to Rats for Twenty-Four Weeks.—Since minimal liver pathology occurred in rats administered Bentyl orally for eight weeks in massive doses of 100 and 500 mg./Kg. of body weight, another experiment was conducted to determine the effects of Bentyl in comparatively large daily doses administered for a period of twenty-four weeks. Two groups of six male and six female rats each were employed. One group received Bentyl hydrochloride by the drug diet method for forty days in daily doses of 15 to 20 mg./Kg. and received an aqueous solution of the drug, prepared fresh daily, in doses of 10 mg./Kg. two times a day, six days a week by stomach tube, for the next one hundred and thirty days. The method of administration was changed because food consumption decreased when drug concentration in the food was increased in order to increase the dose of Bentyl from 15 mg./Kg. to 20 mg./Kg. per day. The other

TABLE IV.—INITIAL AND FINAL AVERAGE BLOOD VALUES WITH STANDARD ERROR FOR THE CONTROL AND EXPERIMENTAL ANIMALS ADMINISTERED REPEATED DOSES OF BENTYL HYDROCHLORIDE

Animal	Dose Group, Mg./Kg.	Erythrocytes, Millions per Cu. Mm.		Hemoglobin, Gm./100 Cc.		Hematocrit, Per Cent Cells		Sedimentation Rate, Mm.—One Hour		Leucocytes, Thousands per Cu. Mm.		Granulocytes, %		Icterus Index	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Dog	4, t.i.d.	5.7 ± 0.2	6.3 ± 0.2	13.1 ± 0.7	11.8 ± 0.6	39 ± 1.6	44 ± 2.0	10 ± 2.9	6 ± 0.3	14.8 ± 1.4	14.9 ± 1.2	67 ± 3.8	68 ± 2.3	4.3 ± 1.0	3.7 ± 0.7
	10, t.i.d.	6.1 ± 0.4	6.7 ± 0.4	14.4 ± 0.7	15.0 ± 0.8	42 ± 1.6	45 ± 2.4	9 ± 2.9	8 ± 2.9	13.1 ± 1.8	13.0 ± 1.7	71 ± 2.6	71 ± 2.2	2.0 ± 0.7	2.5 ± 0.6
	Control	5.8 ± 0.2	6.2 ± 0.2	14.0 ± 0.4	14.8 ± 0.5	41 ± 0.6	44 ± 1.0	20 ± 1.5	9 ± 2.3	18.0 ± 1.9	15.6 ± 0.7	70 ± 3.3	68 ± 3.9	1.5 ± 0.2	2.5 ± 0.4
Rat, 8 weeks	20	7.8 ± 0.3	8.7 ± 0.1	13.8 ± 0.7	15.0 ± 0.3	.....	.....	.....	.....	19.3 ± 1.5	19.3 ± 1.2	17 ± 2.5	16 ± 1.1	.....	.....
administration	100	7.9 ± 0.3	8.3 ± 0.1	13.2 ± 0.5	14.8 ± 0.6	.....	.....	.....	.....	19.0 ± 1.7	19.0 ± 1.0	16 ± 2.3	17 ± 1.6	.....	.....
	500	7.3 ± 0.2	8.6 ± 0.1	14.0 ± 0.3	14.5 ± 0.4	.....	.....	.....	.....	19.0 ± 1.8	21.4 ± 1.1	19 ± 2.2	14 ± 1.0	.....	.....
	Control	8.1 ± 0.2	9.1 ± 0.1	13.7 ± 0.5	15.9 ± 0.3	.....	.....	.....	.....	25.8 ± 2.9	21.0 ± 2.4	20 ± 2.4	17 ± 2.0	.....	.....
Rat, 24 weeks	10, b.i.d.	6.6 ± 0.3	7.9 ± 0.1	13.5 ± 0.4	16.2 ± 0.3	.....	.....	.....	.....	24.3 ± 1.1	29.3 ± 2.8	24 ± 3.3	19 ± 2.2	.....	.....
administration	Control	6.5 ± 0.2	8.1 ± 0.2	12.7 ± 0.2	15.6 ± 0.3	.....	.....	.....	.....	20.0 ± 1.7	35.3 ± 2.5	25 ± 3.4	16 ± 2.8	.....	.....
Monkey	4, t.i.d.	5.8	5.7	14.0	14.0	.....	.....	.....	.....	20.0	15.5	35	39	.....	.....
	No. 1 control	4.8	5.2	13.5	12.5	.....	.....	.....	.....	16.0	15.0	40	33	.....	.....
	No. 2 control	4.5	4.5	12.0	12.0	.....	.....	.....	.....	9.5	12.5	32	45	.....	.....

group of males and females served as a control and was administered water, in volumes equivalent to those used in the drug group, from the fortieth to the one-hundred-thirtieth day of the experiment. All doses were corrected for body weight changes at weekly intervals.

The animals were sacrificed by decapitation after one hundred and seventy days of drug administration. Tissue specimens saved were the same as in the other experiments. These were fixed in Helly's fluid, and paraffin sections were prepared and stained with a trichrome stain.

One of the animals in the control group and one in the experimental group died from intubation of the lungs during the period of drug administration. All other animals appeared normal throughout the experiment. Food consumption determined throughout the experiment, body weight (see Table III), and hematology (see Table IV) of the experimental and control groups were similar. Gross and microscopic pathologic findings for the two groups were similar, were minimal, and were not related to drug administration. The liver changes observed in the previous experiment employing doses of 100 and 500 mg./Kg. a day for eight weeks did not occur in this twenty-four week study.

Repeated Oral Administration to One Monkey.—Bentyl hydrochloride was administered by stomach tube as an aqueous solution to one adult female monkey (*Macaca mulatta*) in doses of 4 mg./Kg., three times a day, six days a week for eight weeks. Two castrate female monkeys served as controls for hematologic studies. No evidence of toxicity was observed. The appetite and activity of the monkey were normal, and it maintained a steady gain in weight. The erythrocyte, total and differential leucocyte counts and hemoglobin concentrations remained in the normal range and did not differ significantly from the values for the controls. No gross pathologic alterations were observed by peritoneoscopic examinations conducted with a Ruddock peritoneoscope, and liver biopsy specimens examined microscopically were normal.

Urinary Excretion.—The method of Richter (8) was employed to determine Bentyl hydrochloride in the urine of experimental animals. The animals were kept in metabolism cages, and urine was collected for periods of twenty-four hours or more. Aliquots were made alkaline and extracted with toluene in separatory funnels on a shaking machine. Aliquots of the centrifuged toluene layer were treated with chloroform and picric acid, and the intensity of the color was read on a Fisher Electrophotometer with a no. 425 filter. The procedure is a general one for the determination of aliphatic amines, and the material measured in the urine may be either Bentyl hydrochloride or a partial breakdown product thereof.

Single doses of 10 mg./Kg. of Bentyl hydrochloride were administered to rats by the oral and intravenous routes. Three groups of four female and four male rats were used for each route of administration and pooled urine samples representing the twenty-four-hour collection from each group were taken for two days. There was no significant difference in the urinary elimination by the two sexes; therefore, results have been combined in the table.

The compound was also given orally in doses of 250 mg./Kg. to male and female rats, and intravenously in 25 mg./Kg. doses to female rats. In these experiments urine samples were collected from individual animals over twenty-four-hour periods.

As shown in Table V, the excretion of Bentyl was roughly 15% or less of the administered doses during the first twenty-four hours. Significant amounts appeared in the urine during the second twenty-four hours after the administration of 25 mg./Kg. intravenously and 250 mg./Kg. orally, but only traces were present after 10 mg./Kg. orally and intravenously. Urine collection was continued for a third day in a number of experiments, but no detectable amounts of amine appeared in these samples.

Doses of 100 mg./Kg. Bentyl hydrochloride were administered orally to three male and three female rats six days a week for three weeks and a dose of 2.5 mg./Kg. was administered intravenously to a

TABLE V.—URINARY EXCRETION OF BENTYL HYDROCHLORIDE FOLLOWING THE ADMINISTRATION OF SINGLE DOSES TO RATS

Route of Administration	Dose, Mg./Kg.	Per Cent of Dose Excreted in Urine: First Day Range	First Day Mean	First Day S. E.	Second Day Range	Second Day Mean	Second Day S. E.
Oral	10	0.0–8.0	3.8 (6)[a]	±1.11	0.0–2.0	0.7 (6)	±0.42
Oral	250	6.2–22.6	9.8 (16)[b]	±1.00	2.2–9.3	5.9 (12)	±0.60
Intravenous	10	3.0–18.0	7.5 (6)[a]	±2.32	0.0–2.0	0.8 (5)	±0.38
Intravenous	25	9.3–13.4	10.7 (3)[b]	±1.30	4.8–7.4	6.1 (3)	±0.80

[a] Values in parentheses represent the number of pooled twenty-four-hour collections from groups of four rats.
[b] These values in parentheses indicate the number of samples from individual rats.

TABLE VI.—URINARY EXCRETION OF BENTYL HYDROCHLORIDE FOLLOWING REPEATED DOSES TO DOGS AND RATS

Animals: Species	Sex	Number	Route of Administration	Daily Dose, Mg./Kg.	Per Cent of Dose Excreted in Urine: Days on Test 1	2	4	9	11	12	18	19
Dog	Male	1	Intravenous	5[a]	6.7	..	2.9	3.8	3.3	..	..	..
Dog	Female	1	Intravenous	5[a]	3.7	..	8.9	4.3	5.8	..	..	..
Rat	Male	3	Oral	100[b]	6.2	6.9	....	....	7.9	7.7	7.0	8.5
Rat	Female	3	Oral	100[b]	11.9	12.2	....	....	14.9	15.6	16.1	16.0

[a] Animals received 2.5 mg./Kg. twice a day, six days a week.
[b] Animals received 100 mg./Kg. once a day, six days a week.

male and female dog twice a day, six days a week for two weeks. Results in Table VI show that excretion was not greatly altered by repeated administration of the compound, and it was within the range obtained after single oral and intravenous doses. Urinary elimination of amines returned to normal within two days after discontinuing drug administration in all experiments.

SUMMARY

The antispasmodic milligram potency of Bentyl hydrochloride on the isolated intestine is approximately one-eighth that of atropine sulfate and ten times that of Trasentine and Pavatrine against the acetylcholine-induced spasm. Its antibarium action is similar to that of other synthetic antispasmodics, and it is similar to Trasentine but weaker than Pavatrine and atropine in relaxing untreated intestine. Bentyl also effectively inhibits the activity of the isolated dog ureter.

In vivo studies show that Bentyl hydrochloride markedly decreases the intestinal motility of anesthetized cats and blocks the intestinal response to acetylcholine. It relaxes the intestinal spasm induced by barium injection in dogs, and small doses relax the intestinal muscle of unanesthetized Thiry-Vella dogs. Oral administration of Bentyl to rats markedly delays the passage of a charcoal meal through the small intestine. The mydriatic action and antisialogogue action of Bentyl is approximately $^1/_{400}$ and $^1/_{800}$ that of atropine.

Bentyl hydrochloride possesses marked topical anesthetic activity. It is relatively nonirritating topically and subcutaneously, and it has low toxicity following single and repeated administrations to animals.

The bulk of Bentyl administered to animals appears to be destroyed in the body since urinary elimination accounts for roughly 15% or less of the drug administered.

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A Further Study of the Reaction of Theophylline with Barbiturates*

By MELVIN F. W. DUNKER

Ten barbiturates, not tested before, and two closely related compounds, ethylphenyl hydantoin and 3,3-diethyl-2,4-dioxytetrahydropyridine, gave no molecular compound with theophylline in alcohol solution. Castrix, 2-chloro-4-dimethylamino-6-methylpyrimidine and the 2-dimethylamino-4-chloro-isomer, likewise gave no molecular compound. Thiophenobarbital gave a reaction product similar to that obtained with phenobarbital and melting at 241–242°.

In a previous publication (1), it was shown that theophylline and phenobarbital in alcoholic solution formed a molecular compound stable to inert solvents but decomposed by dilute hydrochloric acid, dilute solution of sodium hydroxide, and by silver nitrate solution. Nineteen other barbiturates and the closely related diphenyl hydantoin failed to give a molecular compound with theophylline, even though they had very similar groupings on the C_5 atom, e.g., phenyl methyl (Rutonal), phenyl allyl, N′-methylphenylethyl (Prominal).

The present paper reports the behavior of ten additional barbiturates and the closely related compounds, phenylethyl hydantoin (Nirvanol), 3,3-diethyl-2,4-dioxytetrahydropyridine (Pyridione), and Castrix. Pentothal, the sulfur analogue of pentobarbital, failed to give a reaction complex. Homologues of Nostal, namely Pernoston (*sec*-butyl bromallylbarbituric acid) and Sigmodal (*sec*-amyl bromallylbarbituric acid), failed to yield a molecular compound. Barbiturates with cyclic substituents other than phenyl, such as cyclopentenyl (Cyclopal) and cycloheptenyl (Medomin) also failed to react. On the other hand, the 2-thio analogue of phenobarbital yielded with theophylline a crystalline product

* Received January 20, 1950, from the College of Pharmacy, Wayne University, Detroit, Mich.

TABLE I.—SUMMARY OF TESTS

C_5	Barbituric Acid Substituents on C_5	Reactants, Gm. Barbituric Acid	Reactants, Gm. Theophylline	Reactants Recovered, Gm. Barbituric Acid	Reactants Recovered, Gm. Theophylline	Reaction Product, Gm.
O	ethyl *sec*-butyl[a]	0.800	1.494	0.672 (84%)	1.318 (90%)	None
S	ethyl 2-methyl-2-propenyl[b]	0.800	1.401	0.582 (73%)	1.252 (90%)	None
S	ethyl *sec*-amyl[b]	0.800	1.408	0.750 (94%)	1.286 (91%)	None
S	ethyl *iso*-amyl[c]	1.000	1.636	0.905 (91%)	1.514 (93%)	None
S	ethyl phenyl	0.370	0.591	0.011	0.020	0.878 (91%)
O	ethyl cycloheptenyl[d]	1.000	1.580	0.948 (95%)	1.361 (86%)	None
S	allyl *sec*-amyl[c]	0.800	1.246	0.703 (88%)	1.104 (90%)	None
O	allyl cyclopentenyl[e]	1.000	1.692	1.010 (101%)	1.527 (91%)	None
O	bromallyl *sec*-butyl[f]	1.000	1.307	0.927 (93%)	1.196 (86%)	None
O	bromallyl *sec*-amyl[f]	1.000	1.250	0.937 (94%)	1.190 (96%)	None
	Other Compounds					
	Ethylphenyl hydantoin[c]	0.987	1.833	0.811 (82%)	1.617 (90%)	None
	3,3 - Diethyl - 2,4 - dioxytetrahydropyridine[g]	1.000	2.371	0.960 (96%)	2.121 (90%)	None
	2 - Chloro - 4 - dimethylamino-6-methylpyrimidine[h]	1.000	2.310	0.843 (84%)	2.129 (92%)	None

The contribution of samples of barbiturates and other compounds by the following is gratefully acknowledged: [a] McNeil Laboratories; [b] Abbott Laboratories; [c] Parke, Davis & Co.; [d] Geigy Company, Inc.; [e] The Upjohn Company; [f] Ames Company, Inc.; [g] Hoffmann-La Roche, Inc.; [h] W. T. Sumerford.

melting at 242–243°. The complex was decomposed by heating with 10 per cent hydrochloric acid and by treatment with 1 *N* sodium hydroxide solution. Table I lists the barbiturates investigated with the recovery of barbiturate, theophylline, and complex.

EXPERIMENTAL[1]

The calculated amount of U. S. P. theophylline was dissolved in 50 cc. of alcohol (or less for runs containing less than 1 Gm. barbiturate) and the barbiturate was dissolved in the hot solution. Crops of crystals were filtered off, washed, dried in a desiccator, weighed, and the melting points taken. The mother liquor was eventually evaporated to dryness and the residue extracted with ether to separate the barbiturate from the theophylline. The residue after evaporation of the ether was crystallized from dilute alcohol, dried, weighed, and the melting point recorded.

A sample of thiophenobarbital was prepared (2), and when recrystallized from alcohol melted at 217–218°. It gave the following analysis:[2]

Anal.: Calcd. for $C_{12}H_{12}O_2N_2S$: C, 58.04%; H, 4.87%; N, 11.28%. Found: C, 58.16%; H, 4.80%; N, 11.94%.

When 370 mg. of thiophenobarbital was added to the hot solution of 591 mg. theophylline in 20 cc. alcohol, clumps of crystals began to form while the solution was still hot. The first crop of crystals weighed 752 mg. and melted at 241–242°. Upon concentrating the mother liquor a second crop weighing 126 mg. and melting at 238–240° was obtained. The filtrate yielded small amounts of starting materials. The reaction complex when mixed with thiophenobarbital softened at about 200° and melted over the range 205–235°, and when mixed with theophylline softened at about 230° and melted at 240–262°. It appeared to be insoluble in ether, carbon tetrachloride, and cold water. It was slightly soluble in hot water and in chloroform. It was somewhat more soluble in acetone, ethyl acetate, isopropyl alcohol, ethyl alcohol, and dioxan.

A sample recrystallized from alcohol, melted at 241–242°, and gave the following analysis:

Anal.: Calcd. for $C_{28}H_{32}O_6N_{10}S$ (based on 1 thiophenobarbital and 2 theophylline): C, 51.31%; H, 4.64%; N, 23.02%. Found: C, 51.29%; H, 4.80%; N, 22.55%.

When 200 mg. of the complex was heated for thirty minutes on a water bath with 15 cc. of 10% hydrochloric acid and worked up as described earlier (1) there was obtained 78 mg. (95%) thiophenobarbital and 100 mg. (85%) theophylline.

The solution of 200 mg. of the complex in enough 1 *N* sodium hydroxide solution to provide 3 equivalents of base for each equivalent of complex, followed by the addition of 10 cc. U. S. P. alcohol, yielded 105 mg. sodium theophyllinate, 80 mg. thiophenobarbital, and about 70 mg. theophylline. The products recovered total more than the amount expected but the removal of water from sodium theophyllinate is difficult.

SUMMARY

1. A molecular complex was obtained from an alcohol solution of 2 moles theophylline and 1 mole thiophenobarbital. It is decomposed into its components by hot 10 per cent hydrochloric acid or by 1 *N* sodium hydroxide solution.

2. Ten other barbiturates and Nirvanol, Pyridione, and Castrix do not form reaction products.

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[1] All melting points uncorrected.

[2] Analyses by A. Elek, Los Angeles, Calif.

The Relationship of Structure and Antibacterial Activity in the Nitrofurans*

By M. C. DODD,† D. L. CRAMER,‡ and W. C. WARD

The study of many furan derivatives suggests certain necessary structural configurations for *in vitro* and *in vivo* antibacterial activity. The specificity of a nitro group in the 5 position of 2-substituted furans in conferring antibacterial activity is well demonstrated. Certain requirements need be met in the nature of the substituent in the 2 position in order to also obtain *in vivo* activity. Chemotherapeutic activity is displayed by a number of semicarbazone and substituted semicarbazone derivatives of nitrofurans containing the necessary carbonyl group in the 2 position. The substitution of other heterocyclic ring systems such as thiophene and pyrrole does not lead to the antibacterial effectiveness of analogous nitrofuran compounds. Attempts to explain or correlate antibacterial activity with structural configuration by physicochemical methods have not met with success. Certain suggestions as to the mode of antibacterial action of the nitrofurans have been made.

A SURVEY of the literature on the antibacterial action of simple furan derivatives (1) revealed that, with few exceptions, such as furoic acid, compounds of this type were devoid of bactericidal properties unless modified by the presence of heavy metals or combined with other known antibacterial substances. Thus, the information also contained in this report, that the introduction of a nitro group into simple compounds of furan had conferred bacteriostatic or bactericidal properties on the majority of those studied, was of considerable interest. Two years later, Dodd (2) described the therapeutic effect of one of this group of compounds, 5-nitro-2-furaldehyde semicarbazone (Furacin[1]) on experimental infections in mice with various bacteria and trypanosomes. It was pointed out in this paper that this was the single nitrofuran of this group of compounds possessing *in vivo* antibacterial action and certain suggestions were advanced concerning the nature of the relationship of the structure of nitrofurans to their antibacterial action and also on the unique role of the semicarbazone in producing a therapeutically active compound in this series.

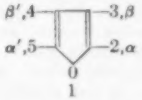
As a result of these studies, an intensive program was carried out in this laboratory and elsewhere concerning the antibacterial action of nitrofuran compounds and the therapeutic action of Furacin. However, in spite of considerable chemical modification and extension on the original compounds reported and the extensive work on the mode of action of several of them (3–5), the original observations have not been markedly changed. It is still impossible to correlate nitrofuran action on bacteria with any particular physicochemical property as was done with the sulfonamides (6, 7) or with acridine compounds (8). Likewise, the particular aspect of the semicarbazone derivative contributing to its singular action is still obscure, although chemical modification has emphasized this type of structure for therapeutic effect.

This report is presented to review and further elaborate on the relationship of the furan ring and various substitutions thereon to the antibacterial and therapeutic properties of these derivatives. The range and type of derivatives studied establish fairly well the essential configurations necessary and there are presented here several factors seemingly inherent in the actions described. The results are not presented as final and we are aware that some of the work still in progress (9) may in the future require either change or addition to these concepts. We are also indebted to those still pursuing the problem for valuable contributions.

All chemical, bacteriological, or pharmacological methods employed to obtain the data presented here were presented previously by the authors cited and will not be reviewed unless modified.

IN VITRO ANTIBACTERIAL ACTION OF FURAN COMPOUNDS

Furan is a five-membered heterocyclic ring having the following structure and numbering system:



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The α,α' or 2,5 positions are more reactive than the β,β' or 3,4 positions, and therefore more compounds involving substitution in the former are

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[1] Brand of Nitrofurazone, N. N. R.

presented. A consideration of the effects of the substitution of various chemical groups at the available positions in the furan ring upon antibacterial activity leads to certain definite conclusions.

Effect of Substitution in Position 5.—It was stated by Dodd and Stillman (1) that antibacterial action was conferred on furan and its 2-substituted derivatives by the introduction of a nitro group in position 5. An extension of this first series continued to bear out this statement to include a majority of the nitro compounds studied. Other groups in the 5 position such as —Cl, —Br, —CH_3, CHO, —CH_2Cl, and —CH_2OH have failed to produce similar antibacterial action in the same compounds. The only possible exception is the amino group in position 5, which produced a minimum of antibacterial action in a few of the above compounds when tested in the same manner. Observations on this aspect are limited to a few compounds by the difficulty encountered in synthesis of amino furans.

The typical change in antibacterial activity provoked by substitution in position 5 can be readily demonstrated by selecting a simple furan derivative, 2-methyl furoate, which has no antibacterial action, and noting the effects of substitution. Nitration of this ester yields 5-nitro-2-methyl furoate which is active *in vitro* against both Gram-negative and Gram-positive bacteria. Reduction of this nitro ester yields the amino ester which displays *in vitro* antibacterial activity though of considerably lower order than the nitro compound. This amino ester is one of a few simple, fairly stable, and isolable 5-amino furans. Acetylation of this 5-amino derivative yields 5-acetylamino-2-methyl furoate which is devoid of bacteriostatic activity. The results are shown in Table I.

Effect of Substitution in Position 2.—As previously noted by Dodd and Stillman (1) 2-substituted furan compounds are usually not antibacterial. There were a few exceptions noted, e.g., furoic acid and two furfuryl alcohol derivatives. However, the order of activity and the high concentrations required for this action differ markedly from the 5-nitrofuran compounds and resemble more closely the action cited above in the amino furan compound.

However, when furan is substituted in position 5 by a nitro group, the nature of the group added in position 2 can be varied without adversely affecting the *in vitro* antibacterial activity. There are two exceptions: (*a*) in those cases where the nature of the substituent in position 2 is such as to produce an insoluble derivative, and (*b*) when the group substituted in position 2 is —COOH. In these two cases the antibacterial activity suffers. For the most part the group substituted in position 2 considerably enhances the activity of the nitrofurans. Nitrofurans which are substituted in position 2 with carbonyl groups are the most active of the series by *in vitro* tests. However, a variety of alcohol esters, acid esters, and other groups in position 2 are antibacterial as indicated in Table II.

Unfortunately, no physical or chemical property is displayed by active compounds which would account for the effects described and the general pattern presented is possible only from a study of many more derivatives than are presented here.

Effect of Substitution in the β,β' or 3,4-Positions.—The effect upon antibacterial activity of the substitution of various chemical groups into the β positions of the furan ring has received comparatively little attention. The difficulty encountered in preparing these compounds has discouraged this approach. A few derivatives have been synthesized and tested for *in vitro* bacteriostatic activity.

TABLE I.—THE EFFECT OF SUBSTITUTION IN THE 5 POSITION ON THE BACTERIOSTATIC ACTION OF 2-METHYL FUROATE

Compound R =	Minimum Bacteriostatic Concentration in Mg. Per Cent after 24 and 96 Hours Incubation									
	S. aureus		*S. hemolyticus*		*D. pneumoniae* I		*E. typhi*		*E. coli*	
	24	96	24	96	24	96	24	96	24	96
R—$COOCH_3$	100	100	100	100	100	100	100	100	100	100
O_2N—R—$COOCH_3$	5.0	10.0	1.0	10.0	100	100	0.5	1.0	2.5	5.0
H_2N—R—$COOCH_3$	100	100	100	100	100	100	100	100	100	100
CH_3COHN—R—$COOCH_3$	100	100	100	100	100	100	100	100	100	100

There are, then, actually two groups, nitro and amino, when placed in the 5 position of the furan ring that confer *in vitro* bacteriostatic action upon such derivatives. However, the degree of the action of the amino compound is so weak as to be markedly inferior to the nitro type, and shows only in concentrations large enough to have introduced other changes in the testing medium to which the effect might be ascribed rather than directly to the presence of the amino group (1).

A similarity to the above sequence of events is found on introducing *p*-amino and *p*-nitro groups into benzene sulfonamide, which produces marked antibacterial properties where none was present before, and this is in turn removed by acylation of the amino group. The similarity is limited, however, especially in view of the marked action of the *p*-amino benzene sulfonamide.

Obviously the effect of the nitro group, so specific in conferring antibacterial activity in the α position, when placed in a β position was of interest. Derivatives were prepared containing the nitro group in the β position and the effect compared with α-nitro derivatives (Table III). Although one derivative, 3-nitro-2-methyl furoate, displayed slight activity, comparatively it was so meager that it seemed likely that the nitro group placed in the β position did not function in the same respect as in the α position. Other derivatives showed no antibacterial effects by the *in vitro* test.

The paucity of β derivatives is such as to make only qualified deductions possible. However, the few derivatives tested do not hold the promise that was so readily evident in the α-substituted nitro-

TABLE II.—BACTERIOSTATIC ACTION OF SOME 2-SUBSTITUTED NITROFURAN DERIVATIVES

Compound R = O_2N (5-position)	Minimum Bacteriostatic Concentration in Mg. Per Cent after 24 and 96 Hours Incubation											
	S. aureus		*S. hemolyticus*		*D. pneumoniae* I		*E. typhi* I		*E. coli*		*Ps. pyocyanea*	
	24	96	24	96	24	96	24	96	24	96	24	96
R—H	10.0	20.0	5.0	20.0	5.0	10.0	1.25	2.5	1.25	2.5	2.5	40
R—C_2H_5	10.0	40.0	10.0	40.0	10.0	10.0	5.0	10.0	5.0	20.0	40.0	100
R—CH_2OH	5.0	10.0	1.25	10.0	2.5	40.0	0.2	0.5	0.5	0.5	2.5	20
R—CH_2OCH_3	10.0	20.0	5.0	20.0	5.0	20.0	1.0	1.0	2.5	2.5	....	....
R—$CH_2OC—C_2H_5$ (O)	20.0	25.0	20.0	50.0	5.0	20.0	2.5	5.0	10.0	10.0	100	100
R—COOH	100	100	10.0	20.0	100	100	100	100	100	100	100	100
R—$COOCH_3$	5.0	10.0	1.0	10.0	100	100	0.5	1.0	2.5	5.0	....	....
R—$CONH_2$	10.0	10.0	2.5	10.0	1.25	100	0.2	0.2	1.0	1.0	....	....
R—CH=$CHCOOC_2H_5$	1.25	5.0	1.0	5.0	0.5	5.0	1.0	5.0	5.0	5.0	5.0	5.0
R—$COCH_3$	0.5	1.66	0.5	1.66	0.2	1.25	0.5	1.0	1.0	1.0	20.0	20.0
R—$C(CH_3)$=$NNHCONH_2$	1.25	2.5	2.5	3.33	3.33	3.33	1.25	2.5	3.33	3.33	3.33	3.33
R—CHO	1.25	1.25	2.5	10.0	5.0	40.0	1.0	1.0	2.5	2.5	10.0	20.0
R—CH=$NNHCONH_2$	1.0	1.25	1.25	5.0	2.5	20.0	0.5	1.0	1.0	1.25	20.0	20.0
R—CH=NOH	5.0	10.0	2.5	5.0	100	100	0.5	0.5	1.0	1.0	....	....

TABLE III.—BACTERIOSTATIC ACTIVITY OF SOME β-SUBSTITUTED FURANS

Compound	Minimum Bacteriostatic Concentration in Mg. Per Cent after 24 and 96 Hours Incubation									
	S. aureus		*S. hemolyticus*		*D. pneumoniae* I		*E. coli*		*E. typhi* I	
	24	96	24	96	24	96	24	96	24	96
3-Nitro-2-methyl furoate (NO_2, $COOCH_3$)	100	100	100	100	100	100	100	100	100	100
5-Acetylamino-4-nitro-2-ethyl furoate (O_2N, CH_3CONH, $COOC_2H_5$)	100	100	100	100	100	100	100	100	100	100
5-Methyl-4-nitro-2-methyl furoate (H_3COOH, NO_2, CH_3)	Inactive at maximum solubility of 10 mg. per cent									

furans. There are available no compounds substituted in the β position when the two α positions are occupied by those groups which produce highly antibacterial furan derivatives.

Effect of Heterocyclic Ring.—Since furan, with a nitro group in position 5 and various substituents in position 2, displayed marked antibacterial action, the function of the furan ring itself and its dispensability were investigated. Thiophene and pyrrole, the sulfur and nitrogen analogues of furan, were chosen as heterocyclic ring systems that should obviously be substituted for the furan nucleus in the known antibacterially active nitrofurans. These thiophene and pyrrole derivatives were tested and compared with the furan analogues (10).

Since nitrofurans having a carbonyl group in position 2 are highly active agents, similarly substituted thiophene and pyrrole compounds were prepared for testing. Thus, 2-acetothienone and 2-acetyl pyrrole were prepared, nitrated, the 5-nitro derivatives separated from the 4-isomers and these compounds along with their semicarbazones compared with 5-nitro-2-acetofurone and its semicarbazone by *in vitro* antibacterial tests. The results are shown in Table IV. The data show that the thiophene ring can be substituted for the furan ring in a like series of derivatives to yield an antibacterial effect somewhat less than the corresponding furan compound. The pyrrole derivatives, while they display some *in vitro* bacteriostatic action are considerably lower in effect than either the furan or thiophene compounds (10).

The result of substituting heterocyclic rings analogous to furan has been to reveal the furan ring as outstanding in producing derivatives possessing *in vitro* antibacterial activity when the nature of the groups substituted in positions 2 and 5 are similar.

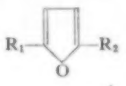
TABLE IV.—COMPARATIVE BACTERIOSTATIC ACTIVITY OF SOME DERIVATIVES OF FURAN, THIOPHENE, AND PYRROLE

Compound	Minimum Bacteriostatic Concentration in Mg. Per Cent after 24 and 96 Hours Incubation											
	S. aureus		*S. hemolyticus*		*D. pneumoniae* I		*E. typhi*		*E. coli*		*Ps. pyocyanea*	
	24	96	24	96	24	96	24	96	24	96	24	96
5-Nitro-2-acetofurone	0.5	1.66	0.5	1.66	0.2	1.25	0.5	1.0	1.0	1.0	20.0	20.0
5-Nitro-2-acetofurone semicarbazone	1.25	2.5	2.5	3.33	>3.33	>3.33	1.25	2.5	3.33	>3.33	>3.33	>3.33
5-Nitro-2-aceto-thienone	2.5	>3.33	1.0	>2.5	1.0	1.25	1.25	2.5	5.0	5.0	>20.0	>20.0
5-Nitro-2-aceto-thienone semi-carbazone[a]	>2.5	>2.5	>2.5	>2.5	2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5	>2.5
5-Nitro-2-acetyl pyrrole	100	100	20	100	>100	>100	40	40	40	40	100	>100
5-Nitro-2-acetyl pyrrole semi-carbazone	Inactive at maximum solubility of 4 mg. per cent											

[a] Maximum solubility in broth = 2.5 mg. per cent.

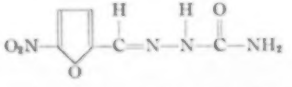
THERAPEUTIC PROPERTIES OF NITROFURAN COMPOUNDS

As originally stated by Dodd (2), the first compound of the series to show therapeutic action when used in experimental infections was 5-nitro-2-furaldehyde semicarbazone (Furacin). As was suggested then, an intensive study of many furan compounds has shown that only nitrofuran compounds containing the grouping —C=N—N—C— in position 2, have displayed similar *in vivo* effect. Thus, the complex necessary to produce the organic molecule for chemotherapeutic activity becomes:



where $R_1 = NO_2$ and $R_2 =$ —C=N—N—C—

Effects of Substitution in —C=N—N—C—.— The initial nitrofuran to display chemotherapeutic activity *in vivo* was 5-nitro-2-furaldehyde semicarbazone as has been pointed out. Structurally this is represented as



Further work on the modification and replacement of the atoms of R_2 showed retention of chemotherapeutic activity but in most cases this effect was lessened. In order to represent graphically substitution in position 2 for *in vivo* activity let $R_2 =$ —C=N—N—C—Y. Activity is present when R_3 = hydrogen, alkyl or hydroxymethyl, R_4 = alkyl or hydroxyalkyl, X = sulfur, oxygen, or imino, and Y = alkyl, hydroxyalkyl, alkoxy, amido, alkylamido, carbamido, carboxyalkyl, guanido, carbamyl, or hydroxyalkyl-carbamyl. There is, then, presented a series of chemotherapeutically active nitrofurans dependent upon two factors: (*a*) the presence of the nitro group in position 5 and (*b*) a substituent in position 2 equivalent to some modification expressed as R_2. An outstanding member of the series in spite of the synthetic modifications so far made remains 5-nitro-2-furaldehyde semicarbazone placing this compound in a position in the nitrofuran series analogous to that enjoyed by sulfanilamide among the sulfonamides as being the parent member. No other substitution either in the 5 or 2 position will reproduce this activity of the above structure. Neither is the effect produced by substituting thiophene for furan. Actually, the semicarbazone of 5-nitro-2-acetothienone, the thiophene analogue of an *in vivo* active nitrofuran, was devoid of therapeutic properties as were all other thiophene or pyrrole derivatives tested.

In Table V, a sample is presented of the data employed in assessing chemotherapeutic action of 13 nitrofuran compounds containing one of the configurations in the 2 position just mentioned. The methods used to determine both the toxicity and therapeutic activity are the same as presented previously by Dodd (2). The figures presented here serve to show samples of data on which the above observations are made and no attempt has been made to indicate relative effectiveness of the compounds except where it is obvious. More precise analyses of signifieance are required. Also, the data are only on infections with *S. hemolyticus*. Some of the compounds are more or less active on other bacterial infections of mice, and one, listed here as ineffective, 5-nitro-2-furaldehyde thiosemicarbazone, is quite active in therapy of infections with *Trypanosoma equiperdum*. However, to include all such data would be too large for the scope of this paper which it is hoped will serve as a guide for future work.

DISCUSSION

The foregoing has pointed out that for effective chemotherapeutic properties in the nitrofuran series, the desirable structural combination has this skeleton:

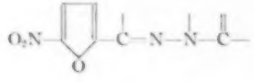
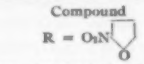


TABLE V.—THE TOXICITY FOR NORMAL MICE, AND THERAPEUTIC VALUE IN MICE INFECTED WITH HEMOLYTIC STREPTOCOCCI, OF SOME NITROFURAN COMPOUNDS

Compound R = 	Toxicity for Normal Mice LD_{50}, Mg./Kg.	Therapy of *S. hemolyticus* Infection in Mice Dose, Mg./Kg.	% Survival	Dosage/LD_{50}
R—CH=NNHCONH$_2$	750	150	67	1/5
R—C(CH$_3$)=NNHCONH$_2$	2000	1000	50	1/2
R—CH=NNHCOCONH$_2$ (CH$_2$CH$_2$OH)	2000	1000	80	1/2
R—CH=NNCONH$_2$	600	300	40	1/2
R—CH=NNHCSNH$_2$	1500	750	0	1/2
(R—CH=NNHC(=NH)—NH$_2$)$_2$·H$_2$SO$_4$	400	200	10	1/2
R—C(CH$_2$OH)=NNHCONH$_2$	1500	750	90	1/2
R—CH=CHC(CH$_2$CH$_2$OH)=NNHCONH$_2$	3000	1000	30	1/3
R—CH=NNHCOCONH (Cl)	3000	1500	70	1/2
R—CH=NNHCOCH$_2$N(CH$_2$CH$_3$CH$_3$)	2000	1500	27	3/4
R—CH=NNHCOCH$_3$	1500	750	15	1/2
R—CH=NNCONH$_2$ (CH$_3$)	500	250	10	1/2
R—CH=NNHCONHCH$_3$	200	100	15	1/2

What, then, are the properties conferred by such an aggregation of structural units which make the net result inimical to a variety of bacteria?

From a purely chemical viewpoint no functional property emerges as a key to this activity. With the exception that the nitro group readily functions as a hydrogen acceptor and is reduced, 5-nitro-2-furaldehyde semicarbazone and substituted semicarbazones are not markedly reactive chemically. The ease of reduction of the nitro group, however, is not limited to the semicarbazone derivatives and thus cannot be considered a key functional property. Also, a comparison of the physical constants of the series has revealed no significant relationship.

Investigations designed to reveal from a biological standpoint the critical processes involved have been carried out. Cramer and Dodd (3) and Cramer (4) have demonstrated that 5-nitro-2-furaldehyde semicarbazone can under specified conditions poise the oxidation reduction potential of growing cultures. This has been interpreted as pointing to electrobiological properties of the molecule as the key factor for effectiveness. The O—R potential data for such organic molecules are beset with many pitfalls in interpretation (11, 12). Polarographic apparent reduction potentials (13) have been used as significant measurements, but an examination of all nitrofurans reveals that for all compounds, this constant falls within a very narrow range (14) revealing no marked deviations in values for *in vitro* and *in vivo* active derivatives. Similarly, examination of a series of nitrofurans based upon relative ease of reduction by a bacterial agency gave inconsistent results (14). Green (5) has pointed out that 5-nitro-2-furaldehyde semicarbazone interferes with glucose utilization, and suggests that the functions of pyridoxal phosphate are involved. 5-Nitro-2-furaldehyde semicarbazone is not antagonized by *p*-amino benzoic acid.

Further observations from the biological data concentrate attention on the nitro group with relation to antibacterial activity and on the necessity of the semicarbazone group for *in vivo* effectiveness. In the former, the action of the group is modified, favorably or not, by various groups in the 2 position, and very definitely reduced in the instances where thiophene or pyrrole rings are present instead of furan. In the typical compound—5-nitro-2-furaldehyde semicarbazone—the mode of antibacterial action varies from other substituted nitro compounds as a unique lag phase effect in contrast to a depression of the logarithmic growth rate with the others (3). Thus, the contribution of the semicarbazide portion uniquely affects the antibacterial action. In addition, bacteriostatic action is accompanied, during this lag extension, by a reduction of the nitro group, which upon reaching a critical point is followed by a normal logarithmic growth phase (4). In bactericidal concentrations no such reduction is noted. This correlation of the intact nitro group and maximum action seems to argue against the role of reduction as the principal factor involved.

In the body, the semicarbazide component is entirely unique in conferring antibacterial properties to the blood. Curiously, the nitro group disappears immediately when injected or fed, and the blood from such animals has no *in vitro* antibacterial action, and physical tests dependent on its presence

are negative. These facts hold also for *in vitro* examinations of the blood of animals which are protected from lethal infections. On the other hand, the compounds may be added to shed blood which then shows antibacterial action and the nitro group appears intact (2). The possibility results that the action occurs in the body by some binding effect through the nitro group which is mediated optimally for *in vivo* action only by the agency of the 2-position semicarbazide. In what manner this produces its therapeutic effect is yet obscure. Perhaps it antagonizes an essential amino acid or growth factor, or the reduction of the group preferentially utilizes this essential substance, removing it from access by the bacteria. Reduction to another active compound is unlikely since, were this the fact, *in vitro* antibacterial tests on blood should be positive. In any event, the usual relationship of detectable blood level to therapeutic action does not seem to prevail, and the relatively long dosage intervals which are most effective (twelve to twenty-four hours) would seem to bear this out further.

The weight of evidence to date, obtained mainly with 5-nitro-2-furaldehyde semicarbazone, since it seems to occupy a parent position, indicates that in some fashion, the effective molecules temporarily block an energy transfer necessary in the microorganism as a prelude to cell division. It may well be that the *in vivo* active nitrofurans are competing with a prosthetic group for a specific protein site. Having become attached, and subject to biological reduction, the change in properties of the original molecule, particularly in view of the recognized lability of the amino furans could well cause a reversal of the inhibition. Thus, normal processes could once more take over. Under any such circumstances, in order to obtain a particular biological effect, a critical range would exist for the combined properties such as three-dimensional molecular size, surface properties, electrical charge, availability of a functional group, and relative stabilities of all members involved in any biological transformation.

To date, no method has been discovered which will measure those properties essential to successful performance as a chemotherapeutic agent. Admittedly, the possibilities have not been exhausted. Obvious applications of previous principles have been unsuccessful, without further refinement of detail. A continued search for such methods is being made. The problem is a challenging one, of considerable theoretical and practical significance.

SUMMARY

The introduction of a nitro group into the α position of simple furan derivatives confers bacteriostatic and bactericidal properties upon these compounds.

The substitution of thiophene and pyrrole, the sulfur and nitrogen analogues, respectively, of furan, for the furan nucleus results in a diminution of antibacterial activity in a series of like derivatives.

Chemotherapeutic activity is obtained by certain nitrofuran derivatives. Semicarbazone and substituted semicarbazone derivatives display therapeutic activity in *S. hemolyticus* and *T. equiperdum* infections. The semicarbazone side chain appears to play a unique role in manifesting *in vivo* activity.

The mode of antibacterial action of 5-nitro-2-furaldehyde semicarbazone (Furacin) is a unique lag phase effect in contrast to the behavior of some other derivatives.

It is not now possible to correlate structural changes with antibacterial activity by physicochemical methods. The relationships that do exist between structure and antibacterial action in the nitrofurans are evident because of a study of many derivatives.

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Digitalis. IX. A Spectroscopic Study of the Baljet Reaction for Digitoxin and Digitoxigenin*,†

By FREDERICK K. BELL and JOHN C. KRANTZ, JR.

Absorption spectra obtained in the region between 460 and 530 mμ strongly indicate that the colored substance formed in the Baljet reaction for digitoxin is not picramic acid but a complex which, on more or less prolonged standing, decomposes in alkaline medium to form picramic acid. Similar results were obtained with digitoxigenin. The results also show that the absorption maximum occurs in the region of 490 to 495 mμ and therefore this region is the most favorable one for analytical purposes.

DURING the course of our studies concerning the Baljet reaction for the cardiotonic glycosides, we became interested in the mechanism of this reaction particularly with regard to the nature of the colored substance formed. It seems almost unavoidable that the reaction depends upon the formation of the picramate ion resulting from the reduction of picric acid in an alkaline medium. Baljet, himself, in his original communication (1) devoted some attention to the colorimetric reaction involved but did not reach any definite conclusions concerning the nature of the colored substance formed.

In an earlier communication (2) we have described a modification of the Baljet reaction using tetraethylammonium hydroxide as the alkali, in which case the color intensity was considerably increased with respect to that obtained when sodium hydroxide was used. If it is assumed that the same fundamental reaction is involved in each of these two procedures, then a spectroscopic study of each method should afford differential evidence concerning the nature of the reaction. This communication is concerned with a report and discussion of the first phase of our spectroscopic observations.

EXPERIMENTAL

From preliminary observations we selected the range between 460 and 530 mμ for examination. The measurements were made with a Beckman Quartz Spectrophotometer, using a tungsten lamp as a light source and absorption cells having a 10-mm. light path. Beginning at the shorter wave length limit, consecutive readings were made at intervals of 2 mμ. The sensitivity of the instrument was adjusted for the point of maximum accuracy as recommended by the maker and the zero adjustment at each particular wave length was made by adjustment of the slit width. Proceeding in this manner a spectrum within the specified range could be scanned in approximately twenty-five minutes.

Our first attention was directed to an examination of the absorption spectra of alkaline solutions of picramic acid, which was obtained from the Eastman Kodak Co. A stock solution of this substance was prepared in absolute methanol and then by proper dilution other desired concentrations were obtained. The two procedures were carried out as follows: In method A, a 2-cc. portion of the picramic acid solution was treated with 2 cc. of a reagent consisting of a mixture of 10 cc. of 2% picric acid and 1 cc. of a 10% solution of sodium hydroxide, which was then diluted to 20 cc. with water. In method B, 1 cc. of the picramic acid solution was treated with 1 cc. of 0.5% picric acid in methanol and 2 cc. of 10% tetraethylammonium hydroxide (Eastman Kodak Co.). Methanol of proper volume was substituted for the test solution in preparing the blanks.

Typical absorption spectra obtained are shown in Fig. 1, in which the optical density has been plotted against the wave length in millimicrons. The indicated concentrations of picramic acid are calculated on the basis of the final concentration in the reaction mixture. The general appearance of the absorption spectra obtained by the two methods is similar. The color intensity is seen to be considerably higher in the case of method B in which there also appears to be a small but definite shift of the absorption pattern toward the shorter wave lengths. With decreasing concentration by either method there is a small but definite and consistent shift in the point of maximum absorption toward the shorter wave lengths, thus suggesting a type of dichromatism. It should be noted that the final reaction mixtures obtained with each method showed high stability in comparison to their respective blanks over a considerable period of time.

Our attention was next directed to the examination of the absorption spectra of digitoxin solutions which had been treated according to method A and method B. A stock solution of digitoxin in absolute methanol was prepared from a sample of the glycoside kindly supplied us by Dr. E. W. McChesney of the Sterling-Winthrop Research Institute. This highly purified sample has been described as sample A by McChesney and his co-workers (3). In examining the digitoxin solutions by method A, the solutions were allowed to stand twenty minutes after the addition of the reagent before the spectrophotometric readings were started, from which point the readings in the region of maximum absorption were made approximately ten minutes later.

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† The expense of this investigation has been defrayed in part by a grant from the Board of Trustees of the United States Pharmacopoeial Convention.

At the left in Fig. 2 are shown the absorption spectra obtained by method A. The digitoxin concentrations are those prevailing in the final reaction mixture. On comparing these spectra with those in Fig. 1 obtained by the same method it is seen that the absorption maximum is considerably broader and there is an increased symmetry in the absorption pattern. It is also apparent that there is a slight shift in the absorption maximum toward the shorter wave lengths with increasing dilution.

For the measurements with solutions obtained by method B, the readings were started immediately after the reagent was added to the digitoxin solution. Proceeding in this manner the readings at the absorption peak were made approximately ten minutes after the addition of the reagent. Representative absorption spectra are shown at the left in Fig. 3, and again the indicated concentrations of digitoxin are those in the final reaction mixture. These spectra compared with the corresponding picramate curves in Fig. 1 reveal some striking differences. The spectra of the digitoxin solutions show a much broader absorption maximum, considerably improved symmetry, and a definite shift in the maximum toward the longer wave lengths. Comparing these curves with those at the left in Fig. 2 obtained by method A, it is apparent that the absorption maximum obtained by method B is not only considerably greater but also considerably broader, thus accounting for the greater sensitivity of the latter method.

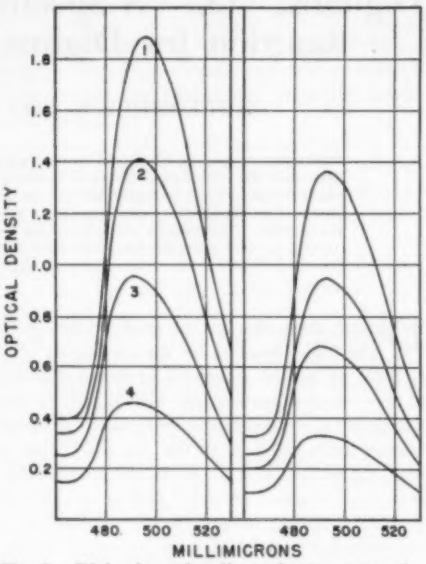

Fig. 2.—Digitoxin and sodium picrate reagent by Method A.

At the left—twenty minutes after additional reagent.

1 = 10 mg. % digitoxin
2 = 7.5 mg. % digitoxin
3 = 5 mg. % digitoxin
4 = 2.5 mg. % digitoxin

At the right—the same solutions after standing six hours.

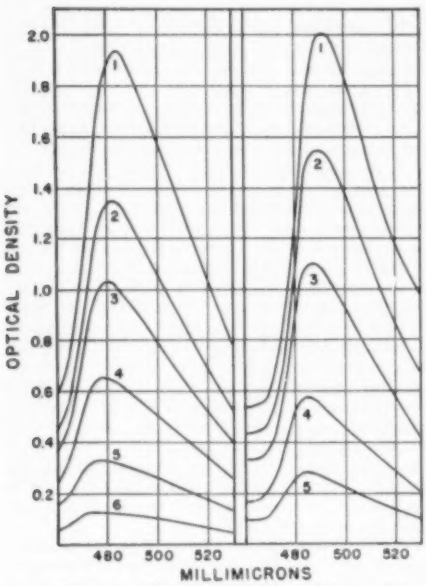

Fig. 1.—At the left—tetraethylammonium picramate.

1 = 75 μg./100 cc.
2 = 50 μg./100 cc.
3 = 37.5 μg./100 cc.
4 = 25 μg./100 cc.
5 = 12.5 μg./100 cc.
6 = 6.25 μg./100 cc

At the right—sodium picramate.

1 = 100 μg./100 cc.
2 = 75 μg./100 cc.
3 = 50 μg./100 cc.
4 = 25 μg./100 cc.
5 = 12.5 μg./100 cc.

Earlier work (4) has shown that in both methods we are dealing with a time reaction in which the color intensity builds up to a maximum followed by a decrease. In method B these changes occur much more rapidly. It therefore seemed desirable to examine the absorption spectra of these solutions after standing various periods of time. In Fig. 2 at the right are shown the absorption spectra of the solutions shown at the left after standing six hours. No temperature controls were used during this period, so that the quantitative aspects of the curves are not to be interpreted too rigidly. The general appearance of these curves is strikingly similar to those measured twenty minutes after the addition of the reagent.

In Fig. 3, the curves obtained by method B shown in the central panel were recorded three hours after the addition of the reagent to digitoxin solution. These curves show definite changes as evidenced by a narrowing of the absorption maximum, a definite decrease in the symmetry and a definite shift in the location of the maximum to the shorter wave lengths. At the right in Fig. 3 the spectra of the four solu-

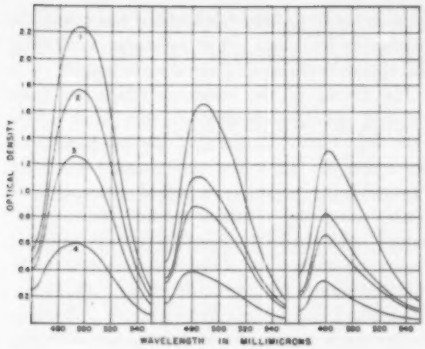

Fig. 3.—Digitoxin and tetraethylammonium picrate reagent by Method B.
At the left—immediately after addition of reagent.
1 = 10 mg. % digitoxin
2 = 7.5 mg. % digitoxin
3 = 5 mg. % digitoxin
4 = 2.5 mg. % digitoxin
Center—same solutions after standing three hours.
At the right—same solutions after standing six hours.

tions are shown after standing six hours. These spectra show a marked similarity to those obtained with the tetraethylammonium picramate as shown in Fig. 1, both in respect to the location of the maxima and the general appearance of the curves. As before, no temperature control was employed in this series of experiments.

Our next experiments were directed to an examination of digitoxigenin solutions in the hope of establishing the effect of the presence or absence of the sugar portion of the glycoside. The sample of digitoxigenin used for this purpose was a portion of a preparation employed in an earlier study (5). The observations were carried out by both method A and method B exactly as in the case of digitoxin except that the concentrations of the solutions of the genin were one-half those employed in the digitoxin experiments. Typical absorption spectra obtained are shown in Figs. 4 and 5.

DISCUSSION

In considering the absorption spectra we have presented, it should be emphasized that these are not true absorption spectra since the systems under examination are not in equilibrium. Spectra obtained by the procedure of method A are probably very similar to the true absorption picture since this system changes relatively very slowly. This is not true in the case of the observations obtained by method B in which the time required for the spectroscopic measurements represents a significant portion of the time in which definite fading of the color takes place. The value of the observations therefore lies in the fact that they were made under comparable conditions.

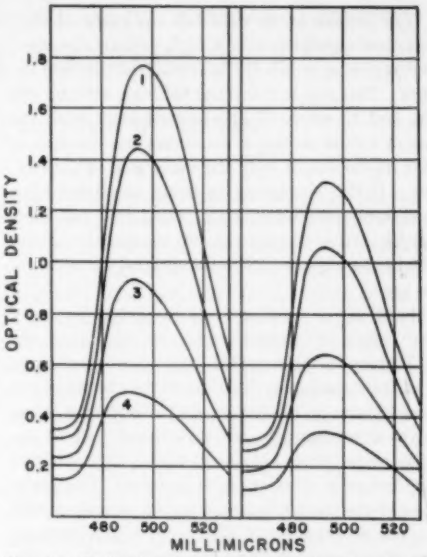

Fig. 4.—Digitoxigenin and sodium picrate reagent by Method A.
At the left—twenty minutes after addition of reagent.
1 = 5 mg. % digitoxigenin
2 = 3.75 mg. % digitoxigenin
3 = 2.5 mg. % digitoxigenin
4 = 1.25 mg. % digitoxigenin
At the right—the same solutions after standing six hours.

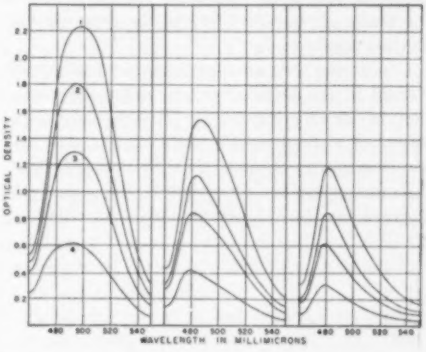

Fig. 5.—Digitoxigenin and tetraethylammonium picrate reagent by Method B.
At the left—immediately after addition of reagent.
1 = 5 mg. % digitoxigenin
2 = 3.75 mg. % digitoxigenin
3 = 2.5 mg. % digitoxigenin
4 = 1.25 mg. % digitoxigenin
Center—same solutions after standing three hours.
At the right—same solutions after standing six hours.

The present study discloses one point of very practical significance in that it reveals the spectral region in which the maximum absorption occurs. This region is located between 490 and 495 mμ and therefore in photocolorimetric work the use of a filter having a maximum transmission in this region would yield the maximum of sensitivity. If the absorption is being measured by a spectrophotometer it is suggested that the wave length setting be made at 492 mμ and the optical system be adjusted to give a spectral slit width of 10 mμ.

In all of our previous work involving the use of the Fisher electrophotometer we have used the 525 green filter supplied with the instrument. The transmission of this filter (6) lies between 493 and 550 mμ and therefore its transmission represents approximately the right-hand half of the absorption curves obtained with the digitoxin and digitoxigenin solutions in this study. This question of the most efficient filter was foreshadowed by the observation of Kedde (7) in his work on the Knudson and Dresbach method (8) for the assay of digitalis preparations. He found that the use of the Zeiss S-50 filter with a principal spectrum line at 494 mμ (9) yielded a maximum sensitivity.

The effect of the sugar portion of the glycoside molecule on the colorimetric reaction is shown by comparing Fig. 2 with Fig. 4, and Fig. 3 with Fig. 5. This comparison reveals that from a qualitative standpoint the two substances yield the same absorption spectra. From a quantitative standpoint the spectra are probably the same within the limits of reproducibility of the experimental method employed.

Concerning the nature of the colored substance formed in the Baljet reaction under the conditions of our experiments, the present results are a source of some disappointment to us. The absorption spectra obtained do show definitely that this substance is not the sodium or the tetraethylammonium salt of picramic acid. If either of these salts is present then there must also be present at least one other substance exhibiting absorption at the same general wave length.

The fading of the solutions which we have followed spectroscopically in this work offers some information concerning the mechanism of the reaction. In a few experiments we have followed this process over a period of twenty-four hours, using the alkaline sodium picrate procedure, and these fragmentary results indicate that a picramate type of absorption is eventually obtained similar to that observed with the tetraethylammonium hydroxide procedure. Pending further study we suggest tentatively the following interpretation of these observations. The colored substance formed under the conditions of our experiments is a complex consisting of the glycoside or genin and some form of the picrate salt (sodium or tetraethylammonium). The intensity of its absorption is considerably higher than that of the picramate salt alone. Its solutions display a type of dichromatism and the complex slowly decomposes (much more rapidly in the case of tetraethylammonium hydroxide) in alkaline medium to form the picramate salt. This colorimetric reaction takes place readily at room temperature in contrast to the usual procedures employed for the reduction of picric to picramic acid. It therefore appears that the degree of specificity of Baljet reaction for the cardiotonic glycosides has been considerably underrated.

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An Investigation of High-Vacuum Freeze Drying as a Means of Drug Preservation. I*,†

By MELVIN A. CHAMBERS‡ and JOHN W. NELSON§

Analogous samples of belladonna leaves were oven dried and lyophilized. Various analytical determinations were made in an effort to determine what differences there are in the two samples as a result of the drying process. A lyophilizing apparatus on a laboratory scale has been developed and is shown in the photograph and in the diagram which has been drawn to scale for the convenience of those who would like to reproduce this for their own use.

A COMPARATIVE study of the effects produced by lyophilizing and oven drying belladonna leaves was made. The object was to determine if the newer method of desiccation, lyophilization, is applicable to belladonna leaves, and to determine what changes, if any, other than desiccation are produced by drying at 50°.

The basis for the activities of plant cells is to be found in their enzymes. The rate at which an enzymatic reaction proceeds is influenced not only by the temperature but also by the length of time the reaction mixture has already been at that temperature. Within certain limits, an increase in the temperature increases the velocity of enzymatic reactions. If plant material is being dried within these limits, these enzymatic reactions will continue for a period of time, thus altering the quantity of the constituents contained therein.

The effect of low temperatures (1) upon enzymic systems has been studied and it was found that the rate was retarded and there was no inactivation of the enzymes which were studied. Attempts have been made to demonstrate hydrolysis in the solid state (2). The rate of hydrolysis was found to be not more than 2 per cent of that occurring in supercooled liquid at the same temperature.

Upon application of these results to the desiccation of plant materials similar effects caused by temperature would be expected. The effect of the temperature used for the drying of belladonna leaves has been noted by Koch (3). It is reported that solanaceous leaves yield 2 per cent to 10 per cent more tropine alkaloids if the leaves are stabilized (4). It was further found that the temperature of drying had a significant influence on the degree of racemization (5). Fermentation of belladonna leaves (6) was carried out at 32° while preventing the loss of water, and upon an alkaloidal determination of the leaf, it was found to contain less alkaloid than the control. It was concluded that enzymes were the causative agents for the loss in alkaloidal content. Thus it can be seen that the temperature of drying affects the alkaloidal content.

A method of drying without the aid of heat is lyophilization. With this method the moisture in the material is frozen and removed by means of reduced pressure directly from the frozen state to the vapor state. It is sublimation of the ice.

This process has been used successfully with the biologicals (7). It was possible to keep convalescent human sera for two or three years while retaining its full potency (8). Antisera and antigens as well as certain related products, when dried in the frozen state and stored *in vacuo*, suffered no deterioration in numerical values and the dried products when reclaimed after a number of years showed no loss in potency. Bacteria and yeasts have been preserved unchanged by lyophilization, and in no instance have the strains shown any changes even in minor particulars (9).

The apparatus that was used in this laboratory is shown in the photograph while in operation and in the accompanying diagram which was drawn to scale. This apparatus has proved to be very satisfactory. Pressures as low as 0.006 mm. of mercury were obtained which kept the material frozen while being dried.

The drying chamber and moisture trap were 4½ and 4 inches in diameter, respectively, with 71/60 ground glass joints. Tubes of this size made it possible to lyophilize several hundred grams of fresh material at one time. The material to be lyophilized was shell-frozen on the sides of the drying chamber so as to expose a maximum of surface area to facilitate drying. The ground glass joints were sealed with Apiezon, a wax (obtained from James G. Biddle Co.,

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Philadelphia, Pa.), which prevented freezing of the joints. The moisture trap was surrounded by a Dewar flask containing dry ice and acetone.

The mercury trap, also surrounded by a Dewar flask containing dry ice and acetone, was included in the apparatus to prevent mercury from the diffusion pump entering the moisture trap and the drying chamber. In order to break the vacuum and to admit dry air into the system a drying tube containing anhydrous calcium sulfate was attached to a stopcock in the system. The McLeod gauge was calibrated and used to measure the pressure in the system. After making the pressure readings, the column of mercury in the gauge was pulled down by means of an aspirator.

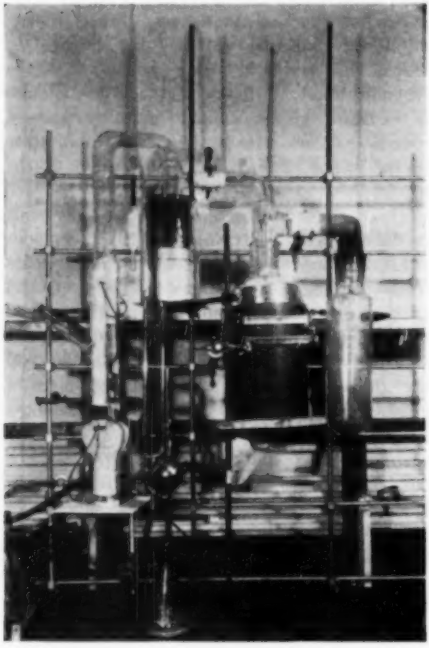

Fig. 1.—Lyophilizing apparatus in operation.

The mercury diffusion pump was included in the system to insure obtaining a pressure low enough to effect lyophilization. A Cenco-Megavac pump was used as a forepump. The arms of the mercury pump were uniformly heated to prevent condensation by wrapping them with asbestos and nichrome wire as a resistor. The water intake and outlet are for the condenser in the pump.

EXPERIMENTAL

The material used for the investigation was the leaves of *Atropa belladonna* L. Opposite leaves were gathered whenever possible to obtain leaves of the same age, size, metabolic processes, and which have been under the same environmental conditions. They were divided into two analogous samples to be oven dried and lyophilized; analyses could be made and the results compared on these samples.

Sample 1A was dried at 65° for twelve hours. It had a dark olive green color. Sample 1B was dried at room temperature under reduced pressure. It had essentially the same color as 1A.

Sample 2A was dried at 45–50° for thirty-six hours and it also had a dark olive green color. Sample 2B was lyophilized. It was very fluffy and occupied a volume more than twice as large as its corresponding oven-dried sample. It had a bright green color.

Samples 3A and 3B were dried in the same manner as 2A and 2B, respectively, and appeared the same in every respect.

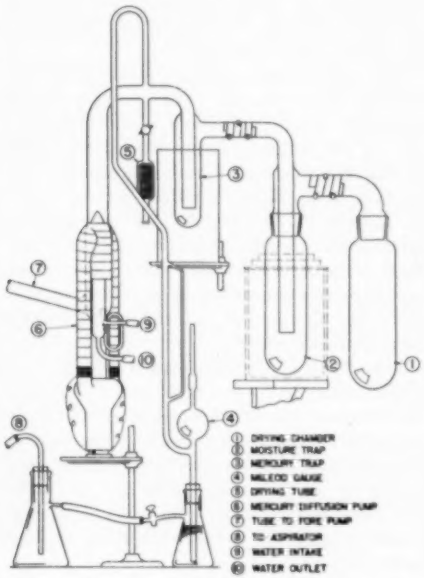

Fig. 2.—To scale drawing of apparatus in Fig. 1.

After drying, the samples were reduced to a No. 40 powder and the various analyses made. The average results in per cent on a dry-weight basis are given in Table I for the various determinations.

The moisture was determined by the U. S. P. XIII method for drugs containing no constituents volatile at 100°. It can be seen that lyophilization reduces the moisture content as low as oven drying at 50° for thirty-six hours.

Ash determinations were made according to the U. S. P. XIII method. The drug is well within

TABLE I.—SUMMARY[a]

	1A	1B	2A	2B	3A	3B
Moisture	5.51	6.24	3.12	3.42	3.88	4.40
Total ash	15.84	15.59	14.59	15.43	15.81	15.45
Acid-insoluble ash	1.46	1.59	0.90	1.34	1.08	1.06
Total alkaloids	...	...	0.328	0.338	0.285	0.307
Total alcoholic extractive	22.33	22.29	23.00	22.97	23.29	25.79
Total nitrogen in extract calculated as protein	0.62	0.53	0.53	0.59	0.79	0.90
Total nitrogen in residue calculated as protein	4.50	4.28	4.53	4.52	4.18	4.51
Amino nitrogen	...	...	0.41	0.35	0.35	0.26
Ammonia and amide nitrogen	...	...	0.054	0.027	0.049	0.015
Ammonia nitrogen	...	...	0.010	0.006	0.018	0.001
Amide nitrogen	...	...	0.044	0.021	0.031	0.014
Starch	...	...	4.81	4.40	5.81	4.85
Sugars	...	...	2.25	3.44	2.31	4.31
Total chlorophyll	2.60	2.50	3.30	4.33	2.45	4.30
Chlorophyll *a*	1.55	1.62	2.09	2.71	1.50	2.64
Chlorophyll *b*	0.98	0.99	1.21	1.61	0.95	1.66
% Chlorophyll *a* of total chlorophyll	59.9	64.8	63.3	62.6	61.4	61.3
% Chlorophyll *b* of total chlorophyll	37.5	37.3	36.7	37.4	38.9	38.7
Animal tests	...	...	...	...	No difference	

[a] Average results of multiple determinations.

U. S. P. XIII requirements for acid-insoluble ash. The leaves were assayed for alkaloids by the U. S. P. XIII method. The data presented here do not indicate any difference, or at least no significant difference, between the lyophilized and oven-dried products. It does not appear, under the conditions of drying here, that the total alkaloidal content is affected by the temperature. It may be that oven drying was carried out so rapidly that no detectable change had taken place.

Total alcoholic extractive was determined to see if heat had any coagulating or other effect. Also because of the greater bulk, it was thought the lyophilized drug would allow greater penetrability resulting in more extractives and quicker complete extraction. The results showed no difference between the two products.

Aliquot portions of the alcoholic extracts were evaporated and total nitrogen, amino nitrogen, and amide nitrogen determined for the purpose of detecting if any proteolysis had taken place. Total nitrogen in the extracts and residues were found to be the same, as well as the amino nitrogen; however, a difference was found in the amide nitrogen. The oven-dried products contained about twice as much as the lyophilized products. This indicates that some proteolysis had occurred during oven drying.

For determining sugar and starch the samples were extracted in a Soxhlet extractor for twenty-four hours with 95% alcohol. The extract was then made up to a 500-cc. volume with 95% alcohol. The residue after extraction was dried at room temperature.

The residue was placed in a beaker with 50 cc. of water and heated on a steam plate for fifteen minutes. After cooling, 20 cc. of a freshly prepared 0.5% solution of pancreatin was added. The mixture was digested in an oven at 55° for one hour and again placed on a steam plate for fifteen minutes. After cooling, the mixture was digested with an additional 20-cc. portion of the pancreatin solution at 55° for one hour. It was then filtered and washed. The filtrate was diluted to 200 cc. and hydrolyzed with 20 cc. of 36% hydrochloric acid in a 500-cc. Erlenmeyer flask on a steam plate for two and one-half hours.

The hydrolyzed extract was partially neutralized with 15 cc. of 30% sodium hydroxide, completely neutralized with solid anhydrous sodium carbonate, and then filtered, washed thoroughly, and diluted to 500 cc. Aliquot portions were analyzed according to the A. O. A. C. methods of analysis (10).

Duplicate results on the same samples treated in the same manner could not be obtained. It was believed the extractions were incomplete. New samples were run in exactly the same manner. The residue, after incubating with pancreatin and washing, was dried; the filter paper and residue were put into a Waring blendor with a small amount of water and broken up. It was then washed into a beaker and treated in exactly the same manner as previously. The second time each sample yielded from one-half to the same amount as it yielded the first time. The combined results are given in Table I. The previous assumption that the extractions were incomplete was correct.

It is possible that some of the cells never came into contact with the extracting medium. Since the material was dried, the tissues would become filled with air. In multicellular particles, it is possible that air would become trapped in the internal cells and thus prevent the extracting medium from entering those cells. This would prevent complete extraction.

The alcoholic extracts were treated and analyzed according to A. O. A. C. methods (11). The carbohydrates were determined according to the Munson and Walker method and calculated as dextrose.

There is a noticeable difference between the samples as a result of the method of drying. The lyophilized samples have a higher sugar content than the oven-dried products. A possible explanation for the difference was through the hydrolysis of starch, but an examination of the results from the starch determinations will immediately eliminate this as a source of the difference. Of the other processes going on in a plant the one most likely to account for the difference in the amount of sugar is respiration. It has been found that sugars are utilized in enzymatic and respiratory processes. In the sample in which these processes were inhibited, the sugar content was greater than in the sample in

which these processes were not inhibited. Lyophilization then preserves the carbohydrates as compared to oven drying at 45–50°.

Chlorophyll was determined according to A. O. A. C. methods (12). It can be seen in the table that the lyophilized product has a greater amount of total chlorophyll and that chlorophyll *a* and chlorophyll *b* occur in the same ratio in the samples which were dried by different methods. It appears that lyophilization prevents some process which has a destructive effect on chlorophyll.

An aqueous extract of both samples was made, saturated with ammonium sulfate, and allowed to set for twenty-four hours. The lyophilized sample had considerably more flocculent precipitate than the oven-dried. The precipitate was filtered, washed into a dialyzing bag, and dialyzed for seventy-two hours with frequent changes of water. The dialyzate was then incubated with a known amount of hyoscyamine sulfate for forty-eight hours and assayed. There was found to be no change in the alkaloidal content, which would seem to indicate the absence of any enzyme having a destructive effect on the alkaloid; however, more tests are necessary before definite conclusions can be made.

Portions of the alcoholic extracts which contained the same amount of alkaloid as determined by chemical assay were evaporated. Water was added, filtered, and the filtrate diluted until it contained 1 part alkaloid to 5000 parts water. These extracts were then tested on the intact eyes of rabbits which had been previously standardized for maximum dilatation with a known solution of atropine. It was found that the action produced by extracts of the lyophilized sample and the oven-dried sample were parallel. This indicates that the proportion of *l*-hyoscyamine to atropine is not altered by the method of drying or is altered to the same degree.

CONCLUSIONS

From the data obtained in this investigation, the following conclusions were drawn:

1. Lyophilization is applicable to the leaves of belladonna as a method of preservation and it is suggested that this method of drying be investigated on other leaves and on other plant organs.

2. Lyophilization reduces the moisture content as low as oven drying at 50° for 36 hours.

3. With respect to the preservation of the alkaloids of belladonna leaf, lyophilization is as effective as oven drying at 50°.

4. Vacuum drying does not facilitate extraction.

5. Oven drying causes more proteolysis than lyophilization.

6. No difference in starch content was found, but extraction of the starch was found to be incomplete and it is suggested that more work be done on extraction problems especially in respect to powder size.

7. Lyophilization preserves the sugars as compared to oven drying at 50°. It is recommended that a similar investigation be carried out on glycosidal- and tannin-bearing drug plants.

8. Oven drying has a destructive effect on chlorophyll while lyophilization preserves this pigment.

9. No evidence was presented in the preliminary tests conducted that the proportion of *l*-hyoscyamine to atropine is different in the two methods of drying.

10. It appears that lyophilization preserves certain constituents better than oven drying at 50°; therefore, it is recommended that lyophilization be adopted as a standard method of drying material which is to be used in any original investigation of these constituents.

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Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods*,†

IV. Apparatus for a New Method Employing Linear Diffusion into Inoculated Agar-Filled Glass Capillaries

By W. W. DAVIS and T. V. PARKE

A description of apparatus designed for the routine large-scale assay of antibiotics employing as a basis linear diffusion in glass capillary tubes is presented. The apparatus described includes the glass capillaries, a device for filling large numbers of them simultaneously with seeded agar, a zone reader for the semiautomatic reading of zone lengths, and an assembly suitable for the rapid cleansing of capillary tubes, test tubes, and bottles. The capillaries used were treated with a water-repellent coating such as ferric stearate which caused the agar column to bond more tightly to the glass.

DIFFUSION procedures for microbiological assay of antibiotics have generally employed Petri dishes or other special dishes with a layer of inoculated agar through which the antibiotic diffuses outward in a ring from a central reservoir of antibiotic solution. This reservoir is sometimes a depression in the agar, sometimes a cup placed on the agar surface, and sometimes a porous disk placed on the agar surface.

In all of these systems the diffusion system involves radial diffusion and a geometry which is not easily described mathematically. This is between linear and two-dimensional diffusion. Further, the dimensionality varies rapidly with the over-all zone diameter. Empirically it has been found, using the porous disk-plate procedure, that over a narrow range of zone diameters corresponding to about a fourfold variation in concentrations of test solutions the zone diameter in a given dish is approximately linear with the logarithm of the concentration of antibiotic. This linear relationship has been widely used in Petri dish diffusion methods of antibiotic testing and the allowed dilution range confined to the linear portion of this empirical curve. In addition, since the duplication of conditions between Petri dishes is poor it is necessary to provide reference zones in each Petri dish and to deal only with differences between the diameters of reference and unknown zones.

Because it appeared possible to better define and control the geometry of the diffusion system and for a number of other reasons including the apparent possibility of improving the efficiency of handling test equipment, it was thought desirable to investigate the possibility of employing linear diffusion into agar-filled glass capillaries. It is anticipated that this diffusion system and some of its potential uses will be described elsewhere.

A method (1) has been established, based on this system, which is believed to be superior in a number of respects to methods employing radial diffusion. Recently it has come to our attention that a method employing linear diffusion in large tubes was described elsewhere (2). Because of the different emphasis placed on efficiency and adaptability to routine testing operations in these two studies, it is felt justifiable to describe the present method in detail.

The present capillary linear diffusion assay method becomes efficient and accurate only as a result of the use of specially developed apparatus for simultaneous or rapid handling of the capillaries. Operations with this special apparatus constitute a major fraction of the work. A description of the apparatus and its operation, therefore, precedes the description of assay methods.

In the organization of work associated with the large-scale use of the test, a somewhat arbitrary choice has been made of a daily unit of work for two persons. This unit involves use of 800 capillaries (and 400 test tubes), one 800-hole capillary filling device, one set of dilution glassware, one constant temperature water bath for incubation, one capillary drying assembly, and one reading device. Only one equipment cleaning cabinet and one oven for drying and sterilizing equipment are necessary at each location where the test is in use.

* Received October 26, 1949, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

† The authors gratefully acknowledge many mechanical design suggestions made by Elmer Lee, and participation in the development of the method for filling capillaries by Edward Fleming.

APPARATUS

Capillaries.—Capillaries to be used in this test are made from Pyrex capillary glass tubing having an outside diameter of approximately 7 to 8 mm. The outside diameter is important only as regards its fit in the filling device and in the test tube. The internal diameter has not been found to be critical and ranges from 0.75 to 1.25 mm.

The capillary is $3^1/_2$ inches long, with one end ground flat and perpendicular to the axis of the capillary. The other end is either lightly fire polished or edge ground to remove the burr.[1]

An adherent water-repellent coating on the capillaries has been found to increase greatly the bonding of agar to the glass surface. In the absence of such coating the agar column has often been found to be pulled out of the capillary on removal from the filling device, or to be displaceable from the capillary with a small amount of pressure. This looseness has resulted in a small per cent of agar plugs being completely displaced after incubation, and a large number of agar plugs being slightly displaced so that they contributed to the spread of zone lengths. When the capillaries are so coated the bonding has been such that the agar plug was not easily displaced even after incubation.

After cleaning the capillaries by soaking in 5% alcoholic KOH and after careful rinsing and drying, the capillaries are coated with an adherent water-repellent film such as ferric stearate.[2] This can be applied by immersion of clean dry capillaries in a solution of 1 Gm. ferric stearate in 1 L. of carbon tetrachloride. After immersion the excess solution is drained off and the capillaries redried in a hot oven. A specially constructed pail with a screen bottom in which about 800 capillaries can be ricked has been found useful for this and other mass handling of the capillaries.

Filling Unit.—The device for filling capillaries with inoculated agar is shown in Fig. 1. It comprises a bowl and a pair of aluminum plates penetrated by 800 closely spaced holes. In order to fill the capillaries 270 ml. of melted seeded agar is poured into the preheated bowl and distributed evenly over the bottom. The pair of plates is placed in the bowl, each of the 800 holes of the bottom plate providing a flat-bottomed well, partially filled with melted agar. The top plate can be slid easily over the bottom plate. This movement is limited by a pair of positioning pins. At one extreme position the holes are in coincidence; in the other extreme position they are several millimeters out of coincidence. When the top plate is moved into a position so that the holes are out of coincidence with those of the bottom plate, the capillaries can be stood in the holes in the top plate. If now the top plate is moved back to bring the holes into coincidence the capillaries all drop simultaneously into the holes in the bottom plate.

When the capillaries are dropped into the well, agar is forced into the capillaries a distance of several centimeters. Since the capillaries stand vertically on the flat bottom surface of the well, the agar is molded flat across the end.

Copper tubing is soldered to the bottom of the bowl permitting heating or cooling by passage of hot or cold water. The bowl and plates are so preheated to 60° before melted inoculated agar is introduced. The plates must be momentarily removed for introduction of the agar. Following dropping of the capillaries, cool water is passed through the copper tubing and the temperature of the entire assembly brought down to 20–30° to gel the agar.

Two threaded handles are provided to screw into holes at opposite edges of the plates. In order to break the solidified agar seal between the plates and the bottom of the bowl, the plate must be turned in the bowl. These handles provided the necessary leverage.

A screw can be turned through a central threaded hole in the bottom plate and bears on the bottom of the bowl. This separates the plate from the bottom of the bowl. When so separated, the capillaries can be withdrawn without displacement of the agar column.

It is necessary for such a diffusion system that the end of the agar column which serves as the initial boundary for diffusion must be sharp and flat, and coincident with the flat end of the capillary; it must remain in this position during and after the moderate temperature changes which the capillaries undergo during filling, incubation, and reading. No difficulties have been observed in securing a substantially perfect molded end on the agar column by this procedure. When the end of a capillary has become chipped slightly at the periphery of the bore, the "flaring" of the agar column at its end can cause diffusion errors. More significant errors arise from any displacement of the agar column in the capillary. Any looseness of the agar in the capillary has been prevented using a water-repellent coating, as described above. Longitudinal thermal contraction of the column can loosen the agar or move the end of the column out of coincidence with the end of the capillary. This is rendered insignificant by avoiding extreme temperature changes and by filling the capillaries to not more than 4 or 5 cm. in height.

When after incubation the capillaries are to be read, the several test tubes containing identical solutions for each sample are removed and the contents—capillaries and solution—are dumped into a cone which permits the solution to drain into a tank. A stream of acetone from a washbottle removes adhering solution and on evaporation leaves the capillaries fairly dry. The operations involved in dumping, drying, and transferring the capillaries to the reader can be carried out without any individual handling.

Zone Reader.—A zone reader of the type previously described (3) for Petri dish methods has been modified as shown in Fig. 2 to read the zones in these capillaries accurately and rapidly. The modifications involved are: (*a*) provision of a new carriage for capillaries, allowing for the rapid removal of each capillary as read and the alignment of the next capillary to be read; (*b*) complete elimination of play between the carriage and the scale by means of a cable suspension; and (*c*) decrease in the pulley size and consequent spreading out of the measuring scale. These details are shown in Fig. 3.

[1] Capillaries made to our specifications have been obtained from the Fisher Scientific Co., Pittsburgh, Pa.

[2] Recent experience has shown that a silicone coating (G-E Dri-Film No. 9977) is superior to ferric stearate and requires less frequent recoating.

Fig. 1.—Capillary filling device with eight-hundred-hole plates removed from bowl.

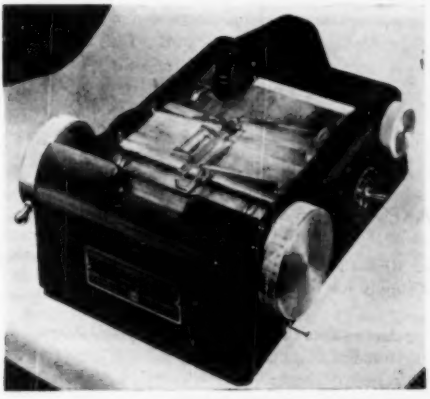

Fig. 2.—Antibiotic zone reader modified for reading capillaries.

The carriage provides for change of the capillaries as read by pushing a camera cable release on the left of the reader. The next capillary to be read rolls into position in the light beam and a second camera cable release on the right side of the reader pushes it forward against a zero alignment stop. No zero setting of the scale is required since the position of the alignment stop and, consequently, of the end of the capillary is adjusted so that their coincident images fall on the reference line on the viewing screen when the scale knob is returned to zero. Each of the two cables on which the carriage is supported passes several times around the $^1/_8$-inch front pulley, then around two ball bearing assemblies, and is drawn taut with a spring. The scale on the $3^1/_2$-inch diameter dial provides easily for direct reading to 0.02 mm.

It is worth noting that the fine detail present in the agar column can be seen with substantially no distortion because the positive cylindrical lens effect of the outer air-to-glass surface of the capillaries is just about equal and opposite to the negative cylindrical effect of the inner glass-to-agar surface.

Cleaning Cabinet.—A device has been designed in order to reduce to a minimum the time necessary for cleansing of the large numbers of capillaries, test tubes, and sample bottles required in this testing procedure, and the 800-hole plates from the filling device. In this device such equipment can be subjected to a blast of high-pressure steam followed by a stream of hot water and finally another blast of steam. A cabinet is provided, in the bottom of which two pipes are arrayed extending from right to left and from which steam and water, respectively, are projected upward through a series of closely

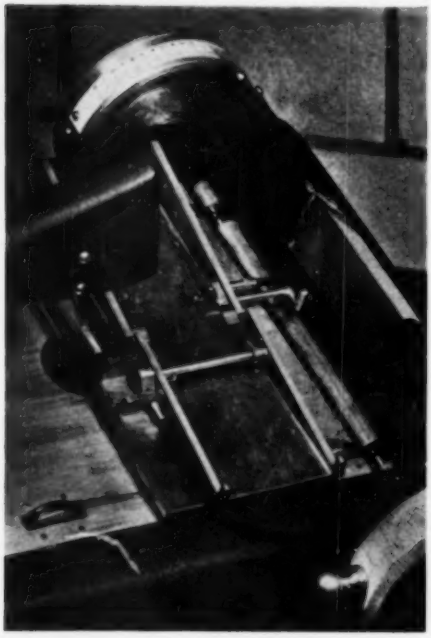

Fig. 3.—Detail view of capillary carriage.

spaced holes. A rack is provided in which the glassware is arranged in an inverted position. The pipes are moved forward and back manually by means of a handle extending through the front of the cabinet. This brings the pipes under all of the glassware in turn. The water leaves through a sink in the bottom of the cabinet; excess steam escapes through several baffled holes at the rear of the cabinet.

The steam supply found satisfactory has a pressure of 40 lb./sq. in. The size of the openings in the steam tube is $^1/_{32}$ inch drilled at $^1/_4$-inch intervals.

After final steam cleaning of the glassware it is placed in a hot-air oven and dried. After drying and before re-use the capillaries are re-coated with ferric stearate as described above.[3]

SUMMARY

Apparatus is described for employing linear diffusion in glass capillaries in routine large-scale antibiotic assay. This comprises the glass capillaries; a device for filling simultaneously large numbers of capillaries with seeded agar; a device for rapid, accurate, and semiautomatic reading of zone lengths; and a device for rapid cleaning of capillaries, test tubes, bottles, etc.

A procedure has been described for treating the glass capillaries with water-repellent coating which causes the agar column to bond more tightly to the glass.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

α,α'-Dihydroxymethyl ether
2,5-Dinitrophenol
3,5-Dinitrophenol
Heptane-1,3-dithiol
Heptane-2,4-dithiol
2-Iodo-3-methylbutane
1,1-Diiodo-2-methyl propane
Benzoguanamine
2-(ρ-Acetaminostyryl)-6-dimethylamino quinolinium methochloride
5-Allyl-5-(1-methylbutyl) barbiturate
2-Amino-4-methylamino-6-ethylamino-1,3,5-triazine
Betaine aldehyde
Ethoxyethyl mercuric chloride
Isopropylmethyl mercuric chloride
Zinc isopropyl xanthate
Solochrome cyanine
1-Methyl-2-mercaptoimidazole
Quercetagetin
Oxyhemoglobin
Soya lipase

Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods*,†

V. A Test Procedure Employing Linear Diffusion into Inoculated Agar-Filled Glass Capillaries

By W. W. DAVIS, T. V. PARKE, W. A. DAILY, LAVELLA M. RUSHTON, and J. M. McGUIRE

Streptomycin, dihydrostreptomycin, and neomycin may be assayed on a large-scale basis by following a procedure employing linear diffusion in agar-filled glass capillaries and using equipment described in the fourth of this series of papers. Directions are given for preparing all required materials, and certain aspects of performance and economics of the method are emphasized.

LINEAR diffusion techniques have been investigated for the assay of penicillin, streptomycin, and other antibiotics. Early in these studies conditions were found by which the assay of streptomycin could be accomplished with efficiency and accuracy. While this paper describes a system suitable for routine assay of streptomycin, dihydrostreptomycin, and neomycin, it is expected that routine assay of penicillin and other antibiotics will be possible by changes in test conditions.

The description of the assay procedure for streptomycin will include composition of the media; preparation of inoculum; dilution of samples; conditions for filling, incubating, and handling capillaries; and a discussion of assay results.

PROCEDURE

Agar Gel.—Agar gel is made by dissolving 4 Gm. of commercial agar per 100 ml. of distilled water. The agar should be dissolved completely by heating in a steam bath or by autoclaving a suspension of the agar. The hot melted agar must be filtered. It has been found convenient to bottle the agar in amounts necessary for one filling operation. This requires about 270 ml. per bottle. The bottles must be autoclaved after filling.

Broth.—Broth is made up as follows: In one liter of water dissolve 8.6 Gm. peptone, 5.6 Gm. casein hydrolyzate, 4.2 Gm. yeast extract, 0.7 Gm. dextrose, 2.0 Gm. dibasic potassium phosphate, 2.2 Gm. beef extract, and 10 ml. 1 *M* NaOH. This broth is bottled with about 500 ml. to 1 L. per bottle and then autoclaved. The *p*H of the broth after autoclaving is 8.0 to 8.1.

Organism Employed.—The organism used in the assay is a strain of *B. subtilis* isolated in these laboratories.[1] Culture bottles containing a layer of nutrient agar are inoculated with a spore suspension. After several days of incubation the spores are harvested with distilled water. This suspension is homogenized in a Waring blendor for two minutes and pasteurized for 30 minutes at 65°. The spore suspension is centrifuged, the supernatant discarded, and the spores resuspended in sterile distilled water. The resulting spore suspension should be diluted to about 50 billion spores per cc. The spore suspension should be bottled in quantities which provide the required inoculum for one week's operation and again pasteurized as above. These operations are carried out in sterile equipment.

The Test.—All samples to be assayed should be diluted with distilled water to approximately two units per cubic centimeter. This dilution with water reduces the concentration of any broth components or nutrients which might be present in the sample. Final one-to-one dilution is made using the broth described above. In making these dilutions it has been found convenient to use automatic burettes and automatic pipettes[2] for delivering distilled water and broth. Dilution schemes have been devised whereby the only other pipettes used are 1-ml. serological pipettes. Dilutions have been made in 60-ml. rectangular screwcap bottles and in stoppered test tubes. The shape of these bottles permits them to be easily and efficiently handled and cleaned. The diluted samples are then poured into the test tubes to be used for the assay. The amount required for each test tube is about 2 ml., the amount being immaterial so long as it is adequate to cover the ends of the capillaries. After all the test solutions have been placed in the test tubes and capped the racks of tubes are placed in the steam cleaning cabinet and are heated in steam for two minutes in order to eliminate contamination. The temperature rises momentarily to 80–90°.

The capillary filling device should be preheated to about 60°. One bottle of agar containing 270 ml. is melted in a steam bath. After cooling the agar to about 60°, 0.5% (1.4 ml.) of spore inoculum is added. The inoculated agar is swirled to thoroughly mix the spores throughout the agar. The

* Received October 26, 1949, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

† The authors gratefully acknowledge enthusiastic participation in the development of test procedures by Mrs. Virginia Boeldt Ward and Mrs. Helen Headley.

[1] This organism was identified by Dr. Nathan R. Smith; Division of Soils, Fertilizers, and Irrigation; Bureau of Plant Industry, Soils, and Agricultural Engineering; Agricultural Research Administration; U. S. Dept. of Agriculture; Beltsville, Md.

[2] Automatic pipettes delivering odd volumes have been made to order by Scientific Glass Apparatus Co.

drilled plates are removed from the filling bowl and the agar poured into the bowl. The agar should be distributed evenly over the surface of the bowl and the drilled plates replaced. The capillaries are now filled as described in the preceding paper. When the filled capillaries are removed from the device they are placed two at a time in each test tube. This arrangement permits the flat bottom of the capillaries to be supported above the round bottom of the test tube, and allows adequate circulation by convection of the antibiotic solution in contact with the end of the agar column. It is not desirable at this stage to inspect each capillary for defects as the unloading procedure should be carried out as rapidly as possible. The test tubes should be capped with aluminum caps or rubber stoppers and incubated overnight in a constant temperature water bath at about 32°. Stainless steel wire racks having places for 6 x 10 test tubes are recommended.

After incubation all of the several test tubes devoted to one sample are removed from the rack at the same time. The capillaries in these test tubes are dumped into a device consisting of a funnel-shaped support placed over a draining screen. This permits the broth to drain from the capillaries while they are held in an upright position. A rinse with acetone removes the broth adhering to the capillaries and on evaporation leaves the capillaries essentially dry. The group of capillaries is then transferred to the capillary zone reader and the lengths of the zones are measured and recorded.

Diffusion of antibiotic into the agar column gives a zone of inhibition (about 3 mm. for a one unit per ml. streptomycin solution). In the region above this zone of inhibition, germination of the spores and a few stages of cell division take place. This growth, however, is limited by the small amount of dissolved oxygen which is available. At the boundary between the zone of inhibition and the zone of oxygen-limited growth a layer of more abundant growth is observed. The organisms in this layer receive a supply of oxygen by diffusion from and through the zone of inhibition below it. The sharpness and density of this layer are quite sensitive to changes in conditions of the assay. Alteration of the medium, inoculum concentration, temperature of incubation, or the antibiotic being tested leads to altered appearance. Contamination of either the agar or the broth solution of antibiotic with fast-growing organisms not inhibited by the antibiotic uses up diffusible oxygen and prevents the formation of a usable layer of growth on which the test depends.

Employing the test conditions described here the layer of growth between the regions of inhibition and of oxygen-limited growth is sharp and dense when streptomycin, dihydostreptomycin, or neomycin is being tested. It is also observed that in the absence of any antibiotic in the test solution a sharp and dense layer of growth appears at the end of the agar column in contact with the broth. Under the same test conditions, when penicillin is being tested the layer of growth between the region of inhibition and of oxygen-limited growth is diffuse and light. Such light and diffuse layers of growth do not provide for easy and accurate measurement of zone length required for large-scale use of this procedure. Conditions are being sought which will give satisfactory layers of growth in penicillin testing.

The zone length is measured from the end of the capillary to the sharp boundary between the zone of inhibition and this layer. These areas in the agar column are shown schematically in Fig. 1.

Curves representing the distribution of zone lengths resulting from assay of a single solution indicate a relatively narrow spread of readings. This distribution of readings suffers if too much time elapses in the unloading of the capillaries from the filling device, or if the temperature of the water bath during incubation varies appreciably from one place in the bath to another. Comparison of the distribution of readings made by the linear diffusion capillary assay method on a single solution with that of other antibiotic assay methods indicates increased reproducibility over the conventional plate-disk method employing Petri dishes, but somewhat lower reproducibility than with the broth dilution technique.

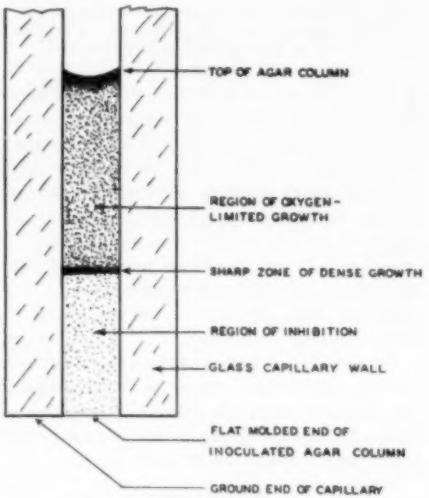
TOP OF AGAR COLUMN
REGION OF OXYGEN-LIMITED GROWTH
SHARP ZONE OF DENSE GROWTH
REGION OF INHIBITION
GLASS CAPILLARY WALL
FLAT MOLDED END OF INOCULATED AGAR COLUMN
GROUND END OF CAPILLARY

Fig. 1.—Schematic cross section of agar-filled capillary with developed zones.

Early experiments in which nutrients were incorporated in the inoculated agar indicated the concentration range over which the zone length varied linearly with the logarithm of the concentration of antibiotic to be of the order of one thousand. The use of agar without nutrients has led to greatly increased slope but has reduced the useful range. The slope of this line is quite sensitive to changes in the medium, *p*H of the broth, and temperature of incubation. Using conditions described above, the slope of the plot of zone length versus logarithm of concentration of streptomycin is about 2.8 mm. per tenfold concentration change. The relationship between zone length and log concentration is essentially linear between 0.2 and 5 u./ml.

In order to determine the standard deviation of assay result per capillary in testing 1 u./ml. solutions seven experiments were made each employing about 100 capillaries. The standard deviations given by

these seven experiments ranged between 0.045 and 0.090 u./ml. or 4.5% and 9.0%. Six similar experiments were made each employing about 50 Petri dishes (100 test zones) in order to determine the standard deviation of assay result per zone in testing 2 u./ml. solutions. Each test zone diameter was corrected by using the average diameter of the two reference zones on each plate. The standard deviations given by these six experiments ranged between 0.146 and 0.190 u./ml. or 7.3% and 9.5%.

Employing the devices which have been designed for handling of capillaries and other equipment enables a team of two persons to handle about 800 capillaries per day. This is equivalent to about 400 Petri dish units. This team requires no support for cleaning of equipment or preparation of materials. During the still limited experience with the routine use of this method the greatest number of samples assayed per day by two individuals using one unit of equipment has been approximately 75 in addition to the standard curve.

Experience indicates that the first two hours of the day are required for reading and calculating the results of the test begun the previous day and to steam clean the capillaries and test tubes. The last two hours suffice to fill the capillaries with seeded agar, to place them in the test tubes containing the test solutions for overnight incubation, and to clean in the steam cabinet, or otherwise, the filling device and all the dilution glassware. The hours between suffice for the dilution of samples, the drying or coating of the capillaries, and other preparatory activities on daily or weekly schedule.

The requirements of this method for agar and nutrients are considerably less than those of the plate-disk method. The space requirements are likewise much less.

The test procedure which has been presented represents one set of conditions which provides a useful test for streptomycin, dihydrostreptomycin, and neomycin. It is likely that a somewhat more extensive survey of important variables will provide conditions for improved accuracy, and that changes in broth and agar composition will allow extension of this method to the testing of penicillin.

SUMMARY

A procedure is described employing linear diffusion in agar-filled glass capillaries which is suitable for large-scale assay of streptomycin, dihydrostreptomycin, and neomycin.

Procedures are given for preparation of all necessary materials.

Some aspects of performance and economies of this method are indicated.

The Effect of Radical Substitution on the Optical Properties of Some Antihistaminics*

By GEORGE L. KEENAN† and THOMAS J. HALEY‡, §

The effect of radical substitutions upon the optical properties of three antihistamine drugs has been determined. The characteristics determined reflect the structural similarities of the compounds examined. The substituents involved, each in the same position, include hydrogen, methoxy, and chlorine.

IN CONNECTION with the microscopic study of some crystalline antihistaminics, attention was drawn to the striking similarity existing in the microscopic characters of the three compounds. The cursory examination in ordinary light led one to conclude that the three substances were essentially identical insofar as the form or habit of the crystals was concerned. As will ultimately be shown, the optical crystallographic examination demonstrated the resemblance, at the same time revealing wherein they differed.

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‡ Medical School, University of California at Los Angeles.
§ The authors wish to thank Dr. C. W. Sondern of the White Laboratories, Inc., for the generous supplies of the drugs used in this investigation.

EXPERIMENTAL

Reference to the structural formulas of these three compounds showed that the substitution of certain radicals in one position accounted for differences in some optical properties while others were not markedly affected. For convenience of discussion, the optical properties of the three compounds first will be given, followed by a more detailed analysis of the data as relating to the radical substitutions. The optical crystallographic data are as follows:

194-A.—*In ordinary light* the habit of the substance is rectangular plates, some of which are elongated (apparently orthorhombic). *In parallel polarized light* (*crossed nicols*) the extinction is parallel on the elongated forms, and some of the plates do not extinguish sharply. *In convergent polarized light* (*crossed nicols*) biaxial interference figures showing the plane of the optic axes are rather common, the isogyres appearing at the edge of the microscopic field. Optical sign (−). $2E = 90 \pm 5°$ (obs.). *Refractive indices:* $\alpha = 1.602$, $\beta = 1.631$, $\gamma = 1.640$, all ± 0.002. β and γ are the most frequently occurring indices, β being shown lengthwise and γ crosswise (Fig. 1).

194-B.—*In ordinary light* this substance also consists of rectangular plates and elongated forms. *In parallel polarized light* (*crossed nicols*) the extinction is parallel and the sign of elongation is negative, the plates all extinguishing sharply. *In convergent polarized light* (*crossed nicols*) no interference figures were observed due to sharp extinction of the plates. *Refractive indices:* The minimum and maximum refractive indices determined on plates showing maximum double refraction and which may be considered as α and γ for the substance are: $\alpha = 1.602$, $\beta =$ indet., $\gamma = 1.640$ (all ± 0.002), and are shown in the following orientations (Fig. 2).

194-C.—*In ordinary light* the habit is seen to consist of quadratic plates and broad elongated forms similar to those characteristics of 194-A and 194-B. *In parallel polarized light* (*crossed nicols*) the extinction is parallel and the sign of elongation is negative. Some fragments extinguish sharply and others remain bright when the microscope stage is revolved. *In convergent polarized light* (*crossed nicols*) biaxial interference figures, showing the plane of the optic axes, are common. The observed axial angle ($2E$) is very small, 15–20°, the figure closely resembling that of phlogopite (mica). Optical sign (+). *Refractive indices:* $\alpha = 1.602$, $\beta = 1.604$, $\gamma = 1.660$, all ± 0.002. All the indices are readily found on the substance. It is interesting to observe that when the material is mounted in liquid with $n = 1.604$ (β), orange and blue borders surround many of the fragments in one orientation, the fragments in the other orientation being practically matched, indicating that α and β are almost identical.

Fig. 1

Fig. 2

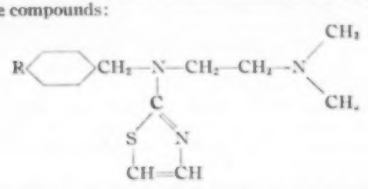
Referring back to the detailed optical crystallographic descriptions of the respective compounds, or, for convenience to the summary in Table I, it will be interesting to observe how these radical substitutions affect the optical characters. As already pointed out, the general habit or form of the crystals is essentially identical for all the compounds, and in this respect they could not be differentiated. By substituting the simple radical H in 194-A, the optical sign is negative, while replacement with Cl in 194-C it is positive. In respect to the observed axial angle ($2E$) there is a very marked difference, the angle being quite large where the H substitution has occurred (194-A) and much smaller when the radical Cl (194-C) is in this position. While biaxial interference figures are readily found in the cases of Cl and H replacements, they are entirely lacking where the methoxy group (CH_3O) has been substituted (194-B).

In respect to the refractive indices, the α value has not been affected by any of the substitutions. In the case of compound 194-C the γ index, 1.660, has been increased from that found to be identical for the other two compounds. The β values where determinable (194-A and 194-C) are appreciably different (1.631 and 1.608). Of interest also is the effect of the substitutions on the orientations in which the commonly occurring refractive indices are found (Figs. 1 and 2).

TABLE I

Sample	Replaced Radical	Habit	Optical Sign	$2E$	Refractive Indices α	β	γ
194-A	H	Plates	(−)	90 ± 5°	1.602	1.631	1.640
194-B	CH_3O	Plates	No figures	.....	1.602	...	1.640
194-C	Cl	Plates	(+)	15–20°	1.602	1.604	1.660

The general formula for these compounds is as follows, the position *R* being the point of substitution of other radicals, as those indicated, for the respective compounds:



For 194-A, R is H, for 194-B, R is CH_3O, and for 194-C, R is Cl. All the compounds have been crystallized from anhydrous ether and are in the form of the hydrochlorides. Their uncorrected melting points as determined with a Melt-Pointer apparatus are 194-A 171° to 172.5°, 194-B 162° to 164° and 194-C 149° to 151°.

SUMMARY

Attention has been called to the effect of radical substitutions upon the optical characters of three crystalline antihistaminics. It has been interesting to observe how closely the optical crystallographic properties reflect the similarity of the compounds as revealed by the structural formulas.

A Chemical Assay for Salts of Dihydrostreptomycin*

By A. A. COLON, G. E. HERPICH, ROSEMARY G. JOHL, J. D. NEUSS, and H. A. FREDIANI

Dihydrostreptomycin salts may be assayed by oxidation with periodic acid in a Kirk distillation apparatus, collecting the evolved formaldehyde in sodium bisulfite and determining it with chromotropic acid reagent. Although the yield of formaldehyde is not stoichiometric, satisfactory results are obtained by standardization of the method by means of pure dihydrostreptomycin salts. A maltol determination is used to correct for any streptomycin present. The results obtained on dihydrostreptomycin trihydrochloride and dihydrostreptomycin sulfate samples are compared with the microbial results.

THE CURRENTLY recommended methods (7) for the analysis of dihydrostreptomycin salts are microbial in nature utilizing either the *B. subtilis* plate assay or the *K. pneumoniae* turbidimetric method. Recently, Conn and Norman (5) have described an amperometric method for titrating both streptomycin and dihydrostreptomycin using trisodium tris-(naphtholazophenyl) carbinol trisulfonate as precipitating agent in triethanolamine citrate buffer.

Dihydrostreptomycin is closely related in structure to streptomycin (10). It is prepared by catalytic reduction of the —CHO group in streptomycin to a —CH_2OH group. Because of this reduction of the aldehyde group, neither the photometric maltol assay (1, 7, 13), nor the direct polarographic assay (12), which have been utilized for streptomycin, is applicable to dihydrostreptomycin. The guanidyl portions of both molecules are identical and may be utilized for chemical assay purposes by modification of the Sakaguchi reaction. This reaction yields similar results with both compounds and has been found to be suitable for assay purposes. Other guanidine derivatives, of course, interfere. However, a satisfactory assay for relatively pure (about 85 to 100 per cent) dihydrostreptomycin salts has been developed in our laboratory (3) using this technique. The streptomycin present may be separately determined by the maltol method (1) and appropriate corrections made.

Strong periodate oxidation of streptomycin should produce one mole of formaldehyde from the primary alcohol group of N-methyl-L-glucosamine portion of the molecule, while for dihydrostreptomycin, in addition to this mole of formaldehyde, a second mole should be produced from the primary alcohol group present in the streptose portion of the molecule. Our laboratory has found this to be true in contrast with the early findings of Lemieux and co-workers (11) who reported that "when the trihydrochlorides of streptomycin and dihydrostreptomycin were oxidized with 1.5 moles of periodate, formaldehyde (0.4 mol. by dimedon reagent) was formed with the latter only . . . ." Apparently, this is due to the fact that our oxidation is carried out at 100°, whereas periodate oxidations are normally performed at room temperature (8). The high temperature evidently results in the hydrolytic production of free N-methyl glucosamine which has the 1-2 glycol structure required for formaldehyde production. Our exploratory experiments showed that periodate oxidation of streptomycin salts, glucosamine, and N-methyl glucosamine produced equal amounts of formaldehyde per mole using the analytical procedure described in this paper. Therefore, it is reasonable to assume that the formaldehyde liberated from streptomycin salts on periodate oxidation comes from the N-methyl glucosamine portion of the molecule. Although the normal periodate oxidation technique for quantitatively estimating dihydrostreptomycin has been developed by Garlock and Grove (4) and since the method used in our laboratory permits estimations in two to three hours elapsed time instead of the twelve to eighteen hours required by the low-temperature oxidation, it was deemed advisable to publish the details of our method.

Although a rigid stoichiometric relationship between the amount of formaldehyde produced and the streptomycin or dihydrostreptomycin taken cannot be readily demonstrated by iodometric titration because of interference by other volatile reducing substances, the use of chromotropic acid as a colorimetric reagent for formaldehyde has resulted in a relatively rapid and precise assay method for dihydrostreptomycin. The specificity of chromotropic acid for formaldehyde has been demonstrated by Eegriwe (6). The yield of formaldehyde from periodate oxidation of dihydrostreptomycin salts was compared

* Received December 8, 1949, from the Chemical Control Division of Merck and Co., Inc., Rahway, N. J.

colorimetrically with formaldehyde solutions of known concentration, and, under the prescribed conditions of analysis, dihydrostreptomycin trihydrochloride was found to produce about 80 per cent, and dihydrostreptomycin sulfate about 70 per cent, of the stoichiometric quantity of formaldehyde. For this reason it is advisable to construct the calibration curve with a dihydrostreptomycin salt of known purity. The procedure herein described has been tested against streptomycin, dihydrostreptomycin, and their mixtures and found to yield reproducible results.

APPARATUS AND REAGENTS

A Kirk micro Kjeldahl apparatus (9) has been found admirably suited for the oxidation and distillation. This is a one-piece glass steam still and condenser which permits rapid quantitative separation of volatile from nonvolatile constituents.

The absorbancies were measured with a Beckman DU Spectrophotometer employing 1-cm. cells, but any filter photometer with a filter transmitting at 570 mμ, or spectrophotometer may be used.

The reagents employed are as follows:

Sodium bisulfite solution 2%, aqueous
Periodic acid solution 20%, aqueous
Chromotropic acid (1,8-dihydroxynaphthalene-3-6 disulfonic acid) reagent 2%, aqueous

The bisulfite and periodic acid solutions are stable for at least one week under ordinary laboratory conditions, whereas the chromotropic acid reagent should be made fresh every other day and stored in the refrigerator.

PROCEDURE

Weigh accurately 1.000 Gm. of sample, transfer to a 50-ml. volumetric flask, make to the mark with distilled water, and mix well. Set up the Kirk distillation apparatus with the receiver end of the condenser slightly below the surface of 20 ml. of 2% bisulfite solution contained in a 125-ml. Erlenmeyer flask. Pipette 2 ml. (40 mg. of dihydrostreptomycin) of the sample solution into the Kirk apparatus and wash down with 2 to 4 ml. of distilled water. Pipette 4 ml. of 20% periodic acid into the apparatus and again wash down with a small quantity of water. Close the sample intake tube with the clamp provided and allow the apparatus to stand for ten minutes with occasional gentle shaking. Place a burner with a low flame under the steam generator and steam-distill for twenty minutes after boiling starts. At the end of twenty minutes, lower the receiver so that the condenser tip is no longer submerged in the bisulfite solution, rinse the tip with a little water, and allow the distillation to proceed for one minute. Remove the flame from the steam generator. Quantitatively transfer the contents of the absorption flask to a 100-ml. volumetric flask, dilute to the mark with 2% bisulfite and mix well. Dilute 5 ml. of this solution to exactly 50 ml. with the bisulfite solution. Pipette 1 ml. of the diluted solution into a test tube cooled in ice water, add 3 ml. (pipette) of concentrated sulfuric acid that has previously been chilled in ice water. Add 2 drops of 2% chromotropic acid reagent, shake to mix thoroughly, and heat in boiling water for twenty minutes. Cool the solution to room temperature and read the absorbancy at 570 mμ against a blank prepared by using 1 ml. of the 2% bisulfite solution in place of 1 ml. of the diluted formaldehyde solution and developing the color with chromotropic acid in the prescribed manner. The weight of dihydrostreptomycin present may then be read from a previously prepared calibration curve. From an independent maltol test (1, 7) the percentage of streptomycin present in the sample is determined and one-half this value subtracted from the percentage found for dihydrostreptomycin.

RESULTS

Samples of dihydrostreptomycin trihydrochloride and dihydrostreptomycin sulfate of 98% purity (after correction for moisture, methanol, and streptomycin content) were used as primary standards. These samples assayed essentially 100% by microbial means. The calibration curves shown in Fig. 1 were prepared by varying the initial weight of sample taken for oxidation. Thus, solutions containing, respectively, 10, 20, 40, 60, and 80 mg. of the dihydrostreptomycin salt were oxidized and the absorbancies plotted against the weights taken. The calibration curve for the sulfate was tested by analysis of a specially purified sample of crystalline dihydrostreptomycin sulfate which was available in small amounts. Quadruplicate determinations gave 98.5, 96.5, 96.0, and 95.0% with an average of 96.5%. This result is consistent with the known 4% loss on drying for this sample when heated for three hours at 100° and less than 5 mm. pressure.

Synthetic mixtures, prepared by mixing the dihydrostreptomycin trihydrochloride used as primary standard with known amounts of streptomycin trihydrochloride, were assayed by the method described above. The recoveries obtained are shown in Table I.

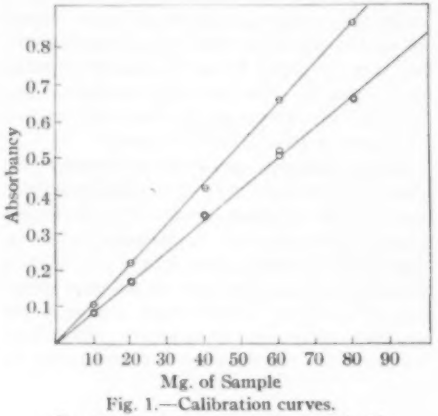


Fig. 1.—Calibration curves.
⊖ Dihydrostreptomycin Hydrochloride
○ Dihydrostreptomycin Sulfate

TABLE I.—ASSAY OF SYNTHETIC MIXTURES OF STREPTOMYCIN TRIHYDROCHLORIDE AND DIHYDROSTREPTOMYCIN TRIHYDROCHLORIDE

—Actual Composition— % Streptomycin Trihydrochloride	% Dihydrostreptomycin Trihydrochloride	—Found— % Dihydrostreptomycin Trihydrochloride	% Recovery
25	75	72.0	96.0
40	60	63.5	106.0
50	50	47.0	94.0
60	40	37.0	92.5
75	25	23.5	94.0
		Av.	96.5

The amount of dihydrostreptomycin trihydrochloride found agrees well, in every instance, with that actually present in the mixture.

The reproducibility of the method in replicate determinations is shown in Table II. The sample was a randomly selected lot.

TABLE II.—REPRODUCIBILITY OF RESULTS

% Dihydrostreptomycin Trihydrochloride	
88.0	89.5
89.0	91.0
87.0	94.0
90.0	89.5
90.0	87.0
Av.	89.5
Av. dev. 1.4	Max. dev. 4.5

As can be seen, the reproducibility is quite good. Since each value reported in this table was obtained on a separate day with different reagent solutions, the spread indicated is probably the maximum to be expected.

Typical results, comparing the chemical and microbial assays, are given in Table III for dihydrostreptomycin trihydrochloride and in Table IV for dihydrostreptomycin sulfate.

Practically all of the results obtained by the microbial method are higher than the corresponding chemical results. It must be pointed out, however, that about 40% of the microbial results are above 100%, which naturally contributes greatly to the difference between the two methods. Furthermore, the microbial assay does not include a correction for streptomycin which may be present to the extent of 0.5 to 1%.

DISCUSSION

The final conditions, as recommended in the above procedure, were selected after many variations had been tried. Ordinary distillation of the oxidized sample solution yielded inconsistent results. Aeration of the sample solution, with and without heating, resulted in exceptionally low yields of formaldehyde. A large excess of periodic acid seemed desirable to provide for the numerous simultaneous reactions taking place that consume this reagent. Therefore, the concentration of periodic acid was varied from a tenfold to a one hundred-fold excess. The amount finally selected was approximately 50 times the theoretical amount required for the reaction being measured. The ten-minute wait-

TABLE III.—CHEMICAL ASSAY OF DIHYDROSTREPTOMYCIN TRIHYDROCHLORIDE

% Dihydrostreptomycin Trihydrochloride Chemical Method	Microbial Assay	Difference in % (Microbial − Chemical)
86.5	100.5	14.0
89.5	97.0	7.5
95.0	97.5	2.5
92.0	98.0	6.0
89.0	101.5	12.5
89.5	102.0	12.5
90.0	96.0	6.0
86.0	101.5	15.5
94.0	99.5	5.5
89.5	100.5	11.0
92.5	103.0	10.5
98.0	98.5	0.5
93.5	91.5	−2.0
92.0	97.5	5.5
95.0	98.5	3.5
92.5	97.5	5.0
95.0	99.5	4.5
93.0	95.5	2.5
87.5	93.5	6.0
91.5	96.5	5.0
89.5	95.5	6.0
91.5	100.5	9.0
88.5	100.0	11.5
91.5	100.5	9.0
93.0	102.5	9.5
89.5	104.5	15.0
	Av.	7.6
	Av. dev.	±3.6

TABLE IV.—CHEMICAL ASSAY OF DIHYDROSTREPTOMYCIN SULFATE

% Dihydrostreptomycin Sulfate Chemical Method	Microbial Assay	Difference in % (Microbial − Chemical)
91.5	96.0	4.5
94.0	105.0	11.0
98.0	97.5	−0.5
93.5	98.5	5.0
96.0	97.5	1.5
96.0	101.5	5.5
95.0	102.5	7.5
97.5	102.5	5.0
97.5	101.5	4.0
97.0	100.0	3.0
91.0	101.0	10.0
95.0	103.0	8.0
91.0	102.0	11.0
91.5	98.5	7.0
98.0	104.5	6.5
92.5	101.5	9.0
94.5	98.5	4.0
94.5	100.0	5.5
92.5	102.0	9.5
96.0	102.0	6.0
96.0	102.0	6.0
93.5	99.5	6.0
95.5	100.0	4.5
	Av.	6.1
	Av. dev.	±2.2

ing period resulted in about a 10 per cent greater yield of formaldehyde than was obtained upon immediate distillation. The use of arsenite in the oxidation flask, as recommended by Boyd and Logan (2) for the determination of serine, did not yield reproducible results. The formaldehyde recoveries by their procedure approximated 15 to 25 per cent of theory.

Since the formaldehyde recovery varied with the nature of the salt oxidized, it was necessary to carry out the calibration with a known sample of the particular dihydrostreptomycin salt to be tested. It was evident that there was some difference in the reaction characteristics for the trihydrochloride and the sulfate because during the oxidation of the trihydrochloride, copious purple-brown iodine fumes formed in the reaction chamber. This did not occur with the sulfate.

In the initial studies a portion of each distillate was analyzed for reducing substances by iodometric titration. These titrations indicated recoveries greater than the theoretical amount of formaldehyde predicted when all of the reducing substances distilled were assumed to be formaldehyde. Prolongation of the distillation beyond the recommended twenty-minute period resulted in the further evolution of relatively small amounts of reducing substances. About a 3 per cent increase was observed for every fifteen-minute increment of additional heating. Formaldehyde was found by the chromotropic acid method to comprise only an insignificant portion of this.

By standardization of the distillation time as well as the concentration of reagents and by use of chromotropic acid reagent for measuring the formaldehyde, satisfactory reproducibility is attained.

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Effect of Pantothenic Acid Antagonists on the Action of Choline Acetylase*

By GUSTAV J. MARTIN, ROBERT BRENDEL, MIRIAM GRAFF, and J. M. BEILER

The effects of several pantothenic acid analogues have been tested on the action of choline acetylase. The only one found to produce a nonspecific inhibition was salicyloyl-β-alanide. Other pantothenic acid analogues were without appreciable effect.

In 1943 the enzyme choline acetylase was first isolated and shown to effect the synthesis of acetyl choline from choline and acetate (1). Intensive research on this enzyme led to the finding, independently by Nachmansohn (2) and by Lipmann (3), that it was associated with a coenzyme. Although the structure of this coenzyme has not yet been completely elucidated, it has been shown (4) to contain pantothenic acid, associated in some way with an adenine nucleotide.

The fact that the coenzyme (coenzyme A) contained pantothenic acid suggested that agents known to act as antagonists to this vitamin in bacterial and other systems might show the same property in relation to coenzyme A, and that this would result in an inhibition of the action of choline acetylase. For this reason a study was undertaken of the effect on choline acetylase of several such pantothenic acid antagonists. This paper presents the results of the investigation.

* Received October 14, 1949, from the Research Laboratories, National Drug Co., Philadelphia, Pa.

EXPERIMENTAL

The measurement of choline acetylase activity was carried out according to the method of Nachmansohn and John (5). Acetone-dried rat brain was used as a source of the enzyme. Additions to the tissue extract were choline chloride, final concentration 0.0025 *M*, sodium acetate, 0.02 *M*, sodium fluoride, 0.02 *M*, and cysteine hydrochloride, 0.015 *M*. It was found that, with the acetone-dried tissue as a source of the enzyme, the reaction proceeded satisfactorily without the presence of physostigmine, so that this was omitted.

After incubation of the reaction mixture for twenty minutes the reaction was stopped (5) and the amount of acetyl choline formed was determined by measuring the contraction produced on the isolated guinea-pig intestine by aliquots of the reaction mixture, and comparing it with the contraction produced by known amounts of acetyl choline. The omission of physostigmine made possible the use of the guinea-pig intestine, which was found to be more sensitive and more convenient to work with than frog muscle. In each experiment controls were run without the addition of any pantothenic acid antagonist, and with added antagonist but without adenosine triphosphate, so that no reaction could occur. It was thus possible to determine whether any of the substances tested had any effect on the tissue used for assay, or any effect on the response of the tissue to acetyl choline.

RESULTS

The results are presented in Table I. Figures are in per cent inhibition, calculated on the basis of the difference in the quantity of acetyl choline produced in the controls without addition of an antagonist and that produced in the presence of an antagonist.

TABLE I.—EFFECT OF PANTOTHENIC ACID ANTAGONISTS ON CHOLINE ACETYLASE

Compounds	Inhibition, % 1.0 Mg./Cc.	0.1 Mg./Cc.
Salicyloyl-β-alanide	100	20
Benzoyl-β-alanide	25	..
Diacetyl pantoyl sulfanilamide	0	..
Pantoyltaurine	0	..
Pantoic acid	0	..
Panthydrazide	0	..
Calcium pantothenate	0	..
Salicylic acid	0	..

The results show that with the exception of salicyloyl-β-alanide and, to a lesser extent, benzoyl-β-alanide, none of the compounds tested had any inhibitory effect. Indeed, both pantoic acid and panthydrazide produced a definite but not consistent stimulation. Pantoyltaurine was tested at concentrations as high as 5.0 mg./cc. without having any effect.

In order to determine whether the inhibition produced by the salicyloyl-β-alanide was specific—that is, whether the compound acted by virtue of a specific antagonism to the coenzyme—attempts were made to reverse the inhibition by the addition of both calcium pantothenate and coenzyme A. Several preparations of coenzyme A, of varying potency were made in this laboratory by the method of Kaplan and Lipmann (6), and one was very generously supplied to us by Dr. Morris Soodak of the Massachusetts General Hospital. None of these preparations nor calcium pantothenate reversed the inhibition produced by salicyloyl-β-alanide.

DISCUSSION

It must be concluded that the inhibition produced by salicyloyl-β-alanide is not due to a specific antagonism to the coenzyme. All the other agents tested were completely without effect, although they exhibited to a greater or lesser degree antagonism to pantothenic acid itself in other systems. These findings are in line with the report by Novelli and Lipmann (7) that phenyl pantothenone had no effect on the action of preformed coenzyme A in yeast. This same situation has now been shown to obtain in the choline acetylase system.

Preliminary studies have been carried out with purine analogues, in the same way as those reported here, since the coenzyme has also been shown to contain adenine (4). Results from these have been negative. It would seem that a substance functioning as a specific antagonist of coenzyme A must have a structure of approximately equal complexity to the coenzyme, and that analogues of its component parts are by themselves without effect.

SUMMARY

The effects of a number of pantothenic acid analogues have been tested on the action of choline acetylase. Salicyloyl-β-alanide gave a nonspecific inhibition; the others were without appreciable effect.

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The Absorption and Excretion of a Liquid Polyethylene Glycol*

By C. BOYD SHAFFER, FRANCES H. CRITCHFIELD, and JOHN H. NAIR III

Additional data have been developed on the absorption and excretion of liquid polyethylene glycols. Results are reported of studies involving the administration of PEG 400 both orally and intravenously to human subjects and subsequently determining the rate and extent of excretion in the urine. Single oral doses of PEG 400 were also administered to rabbits and the percentage recoveries from feces and urine determined. Evidence from these and other studies indicates, that ethylene glycol *is not* formed as a metabolite of PEG 400.

THIS investigation is largely supplementary to an earlier report on the absorption and excretion of the solid polyethylene glycols (1). At the time that work was done the analytical method was not believed applicable to the liquid polyethylene glycols; however, further investigation established the fact that polyethylene glycol 400 (PEG 400) could be determined satisfactorily by the method employed with the solid compounds. It was considered desirable, therefore, to enlarge this study of absorption and excretion to include PEG 400 in view of the widespread interest in the use of this polyglycol and its fatty acid esters in foods, drugs, and cosmetics. In addition, this study was extended to other species, not examined in the previous work, and some attempt was made to identify possible intermediary metabolites of the compound.

METHODS

Most of the techniques used in this work and the routine followed in the experiments with human subjects have been described in detail in the paper referred to above (1). Some additions may be noted, however.

Quantitative Determination of Polyethylene Glycol 400.—Two methods were used for this purpose. The first is the gravimetric silicotungstate method developed in this laboratory (2). This procedure was used in all cases excepting the oral doses to rabbits, and three urinalyses with human subjects.

In the studies using rabbits, the polyethylene glycol was determined in urine and fecal samples by the modification of the alkoxyl method described by Morgan (3). The preparation of samples for analysis is as follows: the twenty-four-hour collection of urine was measured, and exactly one-half was placed in an evaporating dish together with 10 Gm. of "Celite" filter aid and taken to dryness on a steam bath. The residue was then pulverized as finely as possible, and transferred quantitatively to the cup of a Soxhlet extractor in which it was extracted continuously for a minimum of eight hours with alcohol-free chloroform. The extract was then concentrated to 100 ml. and stored in a volumetric flask.

* Received December 10, 1949, from the Mellon Institute of Industrial Research, Pittsburgh, Pa.

The entire twenty-four-hour collection of feces was dried in an oven at 65° to 75° for several days. When thoroughly dry, the feces were ground in a mortar until all of the particles would pass at least a 20-mesh screen. The ground sample was then placed in a Soxhlet cup and the procedure from this point on was the same as outlined for the urine residues.

Aliquots of the chloroform extracts of urine and feces were evaporated to dryness in the reaction flask of the ethoxyl apparatus. When the chloroform appeared to have distilled completely, the flask was attached to a water jet pump for about fifteen minutes to ensure complete removal of the solvent. The aliquot taken should be calculated to contain an equivalent of not more than approximately 30 mg. ethoxyl. The maximum volume that can be accommodated at one time in the flask is about 15 ml., so that where the concentration of polyglycol in the extract is expected to be low it is necessary to evaporate several aliquots of this size in order to obtain a convenient amount of ethoxyl for the determination.

A factor of 1.047 was calculated for converting ethoxyl to polyethylene glycol 400, assuming a molecular weight of exactly 400 for the latter.

Estimation of the extent of recovery of an oral or intravenous dose of polyethylene glycol 400 by analysis of urine and feces in this manner involves considerable uncertainty arising both from loss of urine on walls and floor of collecting cages and from incomplete extraction of the polyglycol by chloroform during this step in the procedure. Quantities of polyethylene glycol 400 of the order of 200 mg. added to urine or in aqueous solution could be recovered to the extent of 95% (average of eight trials). Similarly, this amount added to dried, formed feces could be recovered to the extent of 93%. However, it is questionable whether this type of fecal control experiment is a valid representation of the problem of extracting the polyglycol from the formed feces after an oral dose. Therefore, as a further check, several rabbits were killed and samples of the semiliquid contents of the anterior portion of the large intestine were removed. When known quantities of polyethylene glycol 400 were added to these intestinal contents, an average of only 67% of the polyglycol could be recovered in eight trials. Bacterial decomposition of the polyglycol is not a factor in these low results since autoclaving of some of the samples of intestinal contents prior to addition of the PEG 400 did not influence the findings. There-

fore, it is quite probable that the following fecal analyses are as much as one-third low.

Urine and feces from undosed rabbits give a variable but small blank when carried through the analytical procedure.

Estimation of Intestinal Absorption in Rats.—The procedure used in this experiment is the one described by Cori (4), and was employed in previous studies on the solid polyethylene glycols.

Quantitative Determination of Ethylene Glycol.—This procedure is adapted from that published by Corcoran and Page (5). The authors describe this as a method for the determination of mannitol, but it is equally applicable to any compound that is readily oxidized to periodic acid with formaldehyde as one of the products.

EXPERIMENTAL

Urinary Excretion after Doses to Human Subjects.—One gram of PEG 400 in 20 ml. of physiological saline solution was injected intravenously into each of four human subjects, and the amount excreted in the urine was determined at successive time intervals up to twelve hours. These results are given in Table I. Similarly, the urinary excretion as a function of time was determined in two subjects after oral doses of 10 Gm. of the polyglycol, and in a third subject after the ingestion of 5 Gm. These results appear in Table II.

TABLE I.—PERCENTAGE OF TOTAL DOSE EXCRETED IN URINE OF HUMAN SUBJECTS DURING SUCCESSIVE TIME INTERVALS FOLLOWING THE INTRAVENOUS INJECTION OF 1 GM. OF POLYETHYLENE GLYCOL 400

Time Interval	Excretion—Per Cent of Total Injected H.F.S., %	C.P.C., %	C.S.W., %	U.C.P., %
0–15 min.	17.19	16.33	21.76	13.19
15–30 min.	7.68	7.70	9.28	6.18
30–45 min.	7.40	5.28	7.68	4.44
45 min.–1 hr.	4.13	3.94	6.17	3.63
1 hr.–1 hr. 30 min.	4.61	3.89	9.60	5.02
1 hr. 30 min.–3 hr.	10.37	13.11	12.71	9.61
3–8 hr.	16.23	17.37	6.99	14.20
8–10 hr.	4.80	...	2.39	[b]
10–12 hr.	3.27	10.11[a]	1.94	[b]
Total	75.68	77.73	78.52	

[a] Sum of last two periods.
[b] Determinations lost for these intervals.

NOTE: Analyses for data in this table were performed by the gravimetric method. In separate experiments the total amount of polyethylene glycol 400 excreted in the urine during the twelve-hour period immediately following the intravenous injection of 1 Gm. was determined in 3 human subjects using the analytical method of Morgan for ethoxyl determination. The results of these analyses, expressed as per cent of the 1 Gm. dose injected are as follows: Subject C.B.S., 76%; Subject C.P.C., 75%; Subject U.C.P., 80%.

TABLE II.—PERCENTAGE OF TOTAL DOSE EXCRETED IN URINE OF HUMAN SUBJECTS DURING SUCCESSIVE TIME INTERVALS FOLLOWING THE INGESTION OF A DOSE OF POLYETHYLENE GLYCOL 400 (H.F.S. AND U.C.P., 10 GM.; C.B.S., 5 GM.)

Time Interval, Hr.	Excretion—Per Cent of Total Ingested H.F.S., %	C.B.S., %	U.C.P., %
0–1	12.20	15.60	10.32
1–2	11.15	11.55	8.13
2–3	4.75	5.94	5.11
3–4	5.93	8.78[a]	4.26
4–8	9.48	2.13	7.19
8–10	2.05	Lost	1.92
10–12	1.75	0.41	1.13
12–24	1.41[b]	0.52	1.46
Total	48.72	44.93	39.52

[a] This quantity was excreted between three and five hours.
[b] This quantity was excreted between the twelfth and fourteenth hour.

Intestinal Absorption in Rats.—Groups of fasted adult albino rats were dosed with a 25% solution of PEG 400 and the rate of intestinal absorption was determined according to Cori's technique. An absorption coefficient of approximately 68 mg. per hour per 100 Gm. of body weight was calculated from the results. A mean value of 62% of the dose was absorbed in five hours.

Recovery of Oral Doses from Urine and Feces of Rabbits.—Three rabbits were given a dose of 8.5 Gm. of PEG 400 by stomach tube, and three more received a dose of 5.7 Gm. by the same route. Both urine and feces were collected each day following the dose for three successive days in the first case, and for four days in the latter. The excreta were analyzed for polyglycol by the method described above. The results were calculated as percentage recovery of the original dose, and are given in Tables III and IV.

Recovery of Single Intravenous Doses from Urine of Rabbits.—Three rabbits were given by intravenous injection 10 ml. of a sterile saline solution containing 0.4 Gm. of PEG 400. Urine collected up to twenty-four hours following the injection contained 46, 47, and 49% of the dose, respectively. Three other rabbits received 0.75 Gm. of PEG 400 in the same fashion, and urine was collected for forty-eight hours following the dose. Recovery of the injected polyglycol in these cases was 63, 68, and 69%, respectively. These values are not corrected for the 5% loss indicated by control experiments.

TABLE III.—PERCENTAGE RECOVERIES OF AN ORAL DOSE OF 8.5 GM. OF POLYETHYLENE GLYCOL 400 FROM URINE AND FECES OF RABBITS FOR THREE CONSECUTIVE 24-HR. PERIODS FOLLOWING THE DOSE

	Rabbit No. 43		Rabbit No. 67		Rabbit No. 71	
	Urine	Feces	Urine	Feces	Urine	Feces
1st Day	25.04	2.88	35.85	18.85	26.59	19.88
2nd Day	4.41	4.21	5.53	4.04	4.33	2.48
3rd Day	2.13	0.63	2.30	0.57	1.85	0.82
Total	31.58	7.72	43.68	23.46	32.77	23.18
Grand Total	39.30		67.14		55.95	

NOTE: Results corrected for a possible 5% error in the urinalyses and a possible 33% error in the fecal analyses.

TABLE IV.—PERCENTAGE RECOVERIES OF AN ORAL DOSE OF 5.69 GM. OF POLYETHYLENE GLYCOL 400 FROM URINE AND FECES OF RABBITS FOR FOUR CONSECUTIVE 24-HR. PERIODS FOLLOWING THE DOSE

	Rabbit No. 43		Rabbit No. 67		Rabbit No. 71	
	Urine	Feces	Urine	Feces	Urine	Feces
1st Day	12.13	1.57	15.07	4.37	15.68	1.63
2nd Day	3.17	7.09	3.73	2.43	2.40	1.79
3rd Day	1.95	0.60	2.27	0.69	2.10	1.07
4th Day	0.69	1.22	0.48	2.87	0.62	0.98
Total	17.94	10.48	21.55	10.36	20.80	5.47
Grand Total	28.42		31.91		26.27	

NOTE: Results corrected for a possible 5% error in the urinalyses and a possible 33% error in the fecal analyses.

Constant Intravenous Infusion of PEG 400 Solutions to Dogs.—Since the data on the recovery of PEG 400 after oral and intravenous administration to human subjects and experimental animals indicated a substantial amount of metabolic disposal of this polyglycol it seemed important to test this possibility by another line of investigation. For this purpose, three dogs were subjected to a constant intravenous infusion of a 5% solution of PEG 400 in saline. The solution was delivered from a syringe equipped with a motor-driven worm screw, and the technique was essentially that of the constant infusion method for determining renal clearances (6, 7). The rate at which the polyglycol was excreted in the urine was compared with the rate at which it was infused. As a control similar experiments were performed using "Carbowax" compound 4000 which previous work had shown to be metabolized but very slightly if at all. The results showed that whereas an average of 98 mg. of "Carbowax" compound 4000 was excreted for every 100 mg. injected, only 75 to 88 mg. of PEG 400 could be recovered for every 100 mg. infused. These findings, therefore, support the results of recoveries from single doses in demonstrating a significant metabolic destruction of PEG 400.[1]

[1] In studies that are currently in progress on the absorption of PEG 300 and PEG 400 from intramuscular and subcutaneous depots in dogs, we recover approximately 95% of the injected material (2 ml./Kg.) in the urine over a period of forty-eight hours. During the first six hours of these experiments the animals are anesthetized, the bladder is catheterized, and urine flow is augmented by a constant slow intravenous infusion of saline. It would appear, therefore, that the question of metabolic destruction of PEG 300 and 400 has not yet been definitely decided.

Determination of Ethylene Glycol and Related Substances in Plasma and Urine Following Administration of PEG 400.—The fact that the animal body can effect some metabolic change on PEG 400 logically leads to the question of what products may result. Unfortunately the detection of intermediary metabolites of aliphatic compounds, because of their transient existence, is in most instances very difficult. However, it did appear feasible to test the possibility that ethylene glycol might be produced. Accordingly, three dogs were given 2 Gm./Kg. of PEG 400 by stomach tube, and samples of venous blood were drawn at one-half-, one-, two-, and three-hour intervals following the dose. The plasma was analyzed for ethylene glycol, or rather, substances oxidizable by periodate with the formation of formaldehyde ("apparent" ethylene glycol). There is a small and variable blank in both plasma and urine consisting chiefly of glucose, that reacts to this method. In order to determine whether a small amount of glycol present in, or resulting from, the PEG 400 could be detected by the analytical method similar doses were given in which 1% by weight of ethylene glycol was added to the latter, and on another occasion a 1% solution of ethylene glycol in distilled water was administered. The results of this series of experiments appear in Table V.

This "apparent" ethylene glycol was determined in plasma and urine following both the oral and intravenous doses of PEG 400 to human subjects described above. The amounts of reactive substances determined under these circumstances are extremely small and irregular and bear no relation to the absorption and excretion of PEG 400 as de-

TABLE V.—PLASMA LEVELS OF SUBSTANCES MEASURED AS ETHYLENE GLYCOL IN DOGS AT INTERVALS FOLLOWING THE ORAL ADMINISTRATION OF 2 GM./KG. OF THE FOLLOWING MATERIALS OR MIXTURES

Date	Dog No.	Weight, Kg.	Apparent Ethylene Glycol, Mg. per 100 ml. Plasma 0*	0.5 Hr.	1 Hr.	2 Hr.	3 Hr.
A. Dose = 2 Gm./Kg. Polyethylene Glycol 400							
11-30-48	68172	18.7	....	0.36	1.15	0.46	0.27
	56303	13.4	....	0.46	0.56	0.76	0.95
	56304	16.5	....	1.15	1.15	1.20	1.15
B. Dose = 2 Gm./Kg. of a 1% Solution by Weight of Ethylene Glycol in Polyethylene Glycol 400							
12-2-48	68172	18.9	....	2.51	5.33	4.96	4.96
	56303	13.3	....	3.29	4.83	5.98	4.96
	56304	16.2	....	4.11	5.21	6.52	5.98
C. Dose = 2 Gm./Kg. of a 1% Solution by Weight of Ethylene Glycol in Water							
12-6-48	68172	19.0	0.36	2.73	3.18	2.62	2.40
	56303	13.8	<0.01	2.62	3.35	2.84	2.84
	56304	16.9	1.36	3.99	4.65	3.99	3.99

*The zero or blank sample was drawn immediately prior to giving the dose of polyethylene glycol 400.

termined concurrently. This fact leads to the same conclusion as the data in Table IV, namely, that ethylene glycol is not a metabolite of PEG 400.

DISCUSSION

The mean value of 77% recovered in the urine following the intravenous doses of PEG 400 to human subjects is in harmony with the trend exhibited by the solid polyethylene glycols where it was found that 96% of "Carbowax" compound 6000 could be accounted for in similar experiments, whereas only 85% of "Carbowax" compound 1000 could be recovered (1). The smaller the mean molecular weight, therefore, the greater is the susceptibility to metabolic destruction and the greater is the extent of absorption from the gastrointestinal tract. Both of these latter phenomena would seem to be related to the greater diffusibility of the low molecular weights. In studies reported elsewhere (8) it was shown that the ability of the compounds of the polyethylene glycol series to penetrate cellophane membranes diminishes with increasing molecular weight, and that within the body the distribution of the higher members of the series is extracellular, whereas PEG 400 diffuses intracellularly to a considerable extent. Under these circumstances the smaller molecular weight compounds gain access to enzyme systems not encountered by the larger species, and these systems are apparently able to accomplish some destruction of the polyglycol. The terminal hydroxyl groups would appear to offer the most likely point of initial metabolic attack with cleavage of the carbon-oxygen bond occurring as the reaction proceeds along the chain. Although the ether linkage is generally assumed to be exceedingly stable within the animal body, there remains little doubt that it can be broken, both in aliphatic and aromatic compounds (9, 10).

Since the maximum rate of urinary excretion of the polyglycol following oral doses to the human subjects far exceeded the maximum rate achieved after the intravenous injection of 1 Gm., no analysis of the kinetics of absorption was possible according to the scheme employed with "Carbowax" compound 1000 (1).

A consideration of the data given in Tables III and IV on the recovery in urine and feces of oral doses of PEG 400 to rabbits shows a considerable percentage unaccounted for in each case. In Table III, the average total urinary excretion is 36%, fecal excretion 18%, leaving 46% unaccounted for. If it is considered that this missing portion must have been absorbed to have been destroyed, then total absorption amounted to 46 plus 36, or 82%. Likewise, in Table IV the average total urinary excretion is 20%, fecal excretion 9%, and 71% is unaccounted for. Total absorption in this case is therefore 91%. The principal point of consistency in these two experiments is the fact that the absolute amount destroyed in each case is practically the same, 3.9 Gm. and 4.0 Gm., respectively. This would indicate that, above a certain critical level, the material is metabolized at a constant rate, regardless of its concentration within the body, a situation that is known to exist in the case of several other organic compounds that have been studied.

The discrepancy noted between the rate of infusion and the rate of urinary excretion of PEG 400 during the constant intravenous infusions could have occurred should there be extra-renal routes of disposal of the injected material other than metabolic destruction. This is not believed to be the case, however. The most likely nonrenal route under the circumstances would be secretion into the bile. This alternative was investigated in another experiment in which a bile fistula was created in a dog. A length of polyethylene tubing was inserted into the common bile duct and carried through a small incision in the body wall to the exterior where it drained into a small rubber bag. The bile excreted for twenty-four hours subsequent to an oral dose of 2 Gm./Kg. PEG 400 did not contain any significant amount of polyglycol when analyzed by the ethoxyl procedure. A blank determination (animal not dosed) on bile collected over the same length of time yielded the same result.

SUMMARY AND CONCLUSIONS

1. Four separate intravenous injections of 1 Gm. each of polyethylene glycol 400, two oral doses of 10 Gm. each, and one oral dose of 5 Gm. have been given to a series of five adult human male subjects.

2. PEG 400 is excreted readily in the urine when injected intravenously in man. However, because of the greater diffusibility of the smaller molecule, the rate of excretion is not as great as that of the higher molecular weight polyglycols under similar conditions. Of three subjects receiving 1 Gm. each by the intravenous route, an average of 77 per cent was recovered in the urine within the following twelve hours.

3. Of three human subjects receiving oral doses of PEG 400, between 40 and 50 per cent of the dose was excreted in the urine in the course of the following twenty-four hours. Since the data following intravenous administration indicate that approximately 23 per cent of whatever PEG 400 that is absorbed is destroyed, it is concluded that total absorption of oral doses of this magnitude amounts to between 52 and 65 per cent. These figures are in fair agreement with those for the extent of absorption of this material from the rat intestine as determined by Cori's method.

4. Single oral doses of PEG 400 were incompletely recovered from urine and feces of rabbits even when collection of excreta was continued as long as four days following the dose. The percentage of the material unaccounted for was greater in the case of the smaller dose. It is concluded that this deficit represents metabolic destruction, and certain experiments involving constant intravenous infusion of polyglycol solutions are cited in support of this conclusion.

5. Evidence is offered that ethylene glycol is not formed as a metabolite of PEG 400.

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Chemical and Physical Examination of Some Polyethylene Glycols*

By C. BOYD SHAFFER, FRANCES H. CRITCHFIELD, and JOHN H. NAIR III

Pertinent chemical and physical data relating to a series of polyethylene glycols 200 to 10,000, inclusive, have been developed and are reported. Some compounds of the series are distinguished by their precipitability by barium silicotungstate and most of them show an inverse relationship between ultrafilterability and mean molecular weight. From chemical and X-ray diffraction data developed for the polyglycol-barium silicotungstate complex, a hypothesis as to its structure has been deduced.

FOR THE past several years there has been commercially available a series of products known as the polyethylene (polyoxyethylene) glycols and "Carbowax"[1] compounds. These are manufactured through the reaction of ethylene oxide with water, ethylene glycol, or diethylene glycol, the latter materials furnishing functional groups (in this case hydroxyl) for the propagation of the reaction. The process results in a mixture of diols of different chain lengths, in which the numbers of molecules of various sizes are presumed to be represented by Poisson's distribution (1).

Since experimental methods have not thus far yielded accurate information on the composition of high polymers, the characterization of these products is difficult. Flory (1) has deduced theoretical relationships by means of which molecular size distributions can be calculated. In manufacturing practice, the liquid compounds are defined by a mean molecular weight determined by esterification with phthalic anhydride in pyridine, and product specifications embrace a range of plus or minus 5 per cent of the nominal value. The solid compounds ("Carbowax" compounds) are defined both by a freezing point range and by their viscosity at 210° F. A relationship between viscosity and mean molecular weight permits the designation of the solids according to mean molecular weight as is done in the case of the liquid products.

Because of the diverse applications of these materials in foods, drugs, and cosmetics, this laboratory has had occasion to conduct extended toxicological investigations upon them (2, 3). In the course of this work, the need for better chemical and physical characterization of them has become increasingly apparent, not only for the purpose of analysis of formulations involving them, but also for the interpretation of pharmacological properties in relation to molecular weight. A special problem is that of differentiating between individual polyglycols of which the mean molecular weight has been achieved by the process referred to above, and blends in which the same mean molecular weight results from the mixing of two or more individual products. The following is a description of certain investigations that have revealed some differences in the physical and chemical properties of the members of the polyglycol series that afford a partial basis for identifying them under circumstances wherein the determination of other physical properties would not be feasible.

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[1] This is a registered trade-mark name of the Carbide and Carbon Chemicals Corporation. The products available from this source are polyethylene glycols 200, 300, 400, and 600, and "Carbowax" compounds 1000, 1500, 1540, 4000, and 6000. In this paper the term "polyglycols" will be used in reference to all members of this series. This excludes diethylene and triethylene glycols. The abbreviation "PEG" will be used for polyethylene glycol.

METHODS

Combining Ratio with Barium Silicotungstate.—It has been known for some time that the polyglycols form water-insoluble coordination complexes with barium silicotungstate, and this reaction has provided a means for their quantitative determination (4). In the present work, samples of each member of the series have been submitted to this procedure, and the results have been calculated as parts by weight of ignited residue per part of polyglycol. This value is designated as the "combining ratio." The method has been modified slightly in the direction of more complete standardization in order to secure consistent results with polyethylene glycols 200 and 300, in which cases water-solubility of the precipitate is a factor. The total volume of the reacting solutions has been adjusted to 250 ml., and a small amount of a shredded paper filtering aid has been added in order to coagulate the precipitate. During the filtration step, the amount of wash water is limited to 5 portions of 20 ml. each.

The weight of barium silicotungstate ash obtained from equal weights of different polyglycols above polyethylene glycol 300 are nearly constant, and not proportional to the molecular weight of the glycol. In order to investigate the composition of the complex precipitated, and in particular to obtain some rational basis for expecting constant composition, the following experiment was performed. Fifty-milligram samples of each of these polyglycols (20 mg. in the case of "Carbowax" compounds 4000 and 6000 since these complexes are more difficultly filterable) were treated in replicates of 10 with barium silicotungstate, and the precipitate was filtered, dried at 110° for several hours, and weighed. These precipitates, which contain the polyglycol, were then transferred to a muffle at 750° for one hour, following which the barium silicotungstate ash was weighed. The composition of this ignited residue has been verified by chemical analysis as $2BaO.SiO_2.12WO_3$.

X-Ray Diffraction Technique.—The solid polyglycols give distinctly crystalline X-ray diffraction patterns (see Fig. 1), but the liquid compounds are obviously not adaptable to examination by this method. The solid complexes formed by the reaction of the polyglycols with barium silicotungstate, however, afford a convenient means of converting all of these compounds to a form suitable for this purpose. Accordingly, X-ray diffraction powder patterns have been taken of these complexes involving polyethylene glycols 400 and 600, and "Carbowax" compounds 1000, 1540, 4000, and 6000, respectively.

Two types of photographs have been made: (*a*) Debye-Scherrer patterns, and (*b*) monochromatic pinhole patterns. The former were taken on a narrow strip of film circularly disposed in a camera of 114.6 mm. diameter using CuKα radiation and exposures of four hours. The sample was loaded inside a thin-walled paralodion tube of 0.6 mm. i.d. for the exposure. The monochromatic pinhole patterns were prepared with the General Electric vacuum-back flat cassette assembly using a sample-to-film distance of 8 cm. Micronized quartzite was added to each sample in the approximate proportion of 1:1, the reference lines thus obtained permitting an accurate determination of sample-to-film distance. For photographs of this type the sample was held between two plates. The monochromatic pinhole technique gives a greater dispersion of the lines in the pattern, and was therefore employed where measurements of spacings were to be made. The inclusion of quartzite provides an internal standard, since the length of the one important spacing produced by this material is well known.

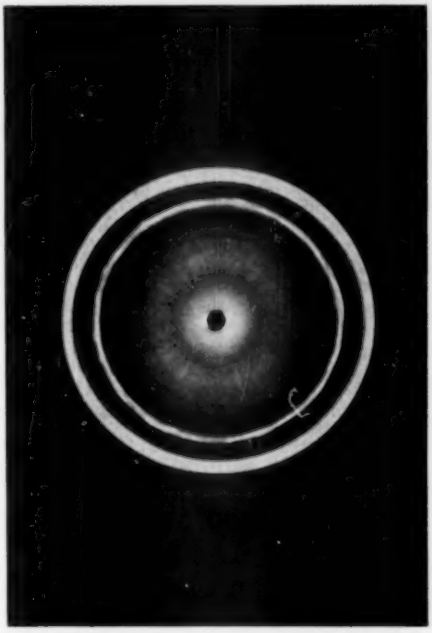

Fig. 1.—Monochromatic pinhole X-ray diffraction pattern of "Carbowax" compound 4000.

Filtration through Cellophane Membranes.—Each of the members of the polyglycol series has been filtered in aqueous solution through cellophane membranes. Two types of membrane have been employed: (*a*) Du Pont cellophane No. 600 PT in sheet form, and (*b*) a seamless cellophane tubing sold by various laboratory supply houses as dialyzer tubing. This latter material is very closely akin to, if not identical with, "Visking" sausage casing. Two forms of apparatus have been used. The first of these is the Coolidge device (5) which consists of a modified centrifuge tube, and in which the filtration pressure is provided by centrifugal force. The second is the Lavietes filter capsule (6) which utilizes the hydrostatic pressure of a column of mercury for the same purpose. Determination of the polyglycol in substrate and filtrate was made according to the precipitation method described above. The percentage ratio of the concentration of polyglycol in the filtrate to that in the substrate is expressed as the "per cent filterable."

Determination of Ethylene Glycol in Polyglycols.—The basis of this determination is the fact that ethylene glycol is oxidized by periodic acid by virtue of the presence of hydroxyl groups on adjacent car-

bon atoms. The polyglycols (including diethylene glycol and above) are unaffected. Approximately 20 Gm. of sample is weighed into a 500-ml. Erlenmeyer flask containing about 50 ml. of 15% (w/v) sulfuric acid. To this there is added, from a burette, exactly 35 ml. of 0.2 *N* periodic acid, and the flask is swirled gently and allowed to stand for about one minute. The sides of the flask are then washed down with 175 ml. of a saturated solution of sodium bicarbonate, and 50 ml. of 0.2 *N* sodium arsenite solution is added from a burette. The solution is allowed to stand ten minutes, with occasional swirling. The excess arsenite is then titrated with standard 0.1 *N* iodine, using a few milliliters of starch indicator. A blank is run following the same procedure but omitting the sample. The percentage by weight of ethylene glycol in the sample is given by the formula $\frac{(A - B)N \times 3.104}{\text{weight of sample}}$, where *A* is the ml. of iodine required for the sample, *B* the ml. of iodine for the blank, and *N* is the normality of the iodine solution used.

RESULTS AND DISCUSSION

Reaction with Barium Silicotungstate.—The results of the investigation on the composition of these complexes are given in Table I expressed in terms of mg. of product per mg. of sample taken. The calculated molar compositions demonstrate the decreasing amount of polyglycol in the complex for increasing molecular weight of the former. However, there is a substantially constant molar content of polyglycol oxygen in the complex, whether the calculation be made in terms of total oxygen, ethoxyl oxygen, or ether oxygen atoms. Averages for the 6 polyglycols are:

Total oxygen, 18.32, standard deviation ±0.34 (1.9%)
Ethoxyl oxygen, 17.52, standard deviation ±0.76 (4.4%)
Ether oxygen, 16.72, standard deviation ±1.40 (8.4%)

The most constant value is obtained when the calculation is made in terms of total oxygen atoms, and it seems logical to believe, therefore, that the bonds forming the complex include every oxygen atom of the polyglycol, whether it be in hydroxyl or ether linkage. In fact, the results are more constant than would be expected because the nominal mean molecular weight was employed in each calculation, whereas manufacturing controls hold the mean molecular weights of the products to tolerances of only ±5% of this value.

The complexes formed by the interaction of the polyglycols with barium silicotungstate become less crystalline as the molecular weight of the polyglycol increases. This observation is founded upon the appearance of these precipitates at the time of their formation, their ease or difficulty of filtration, and their X-ray diffraction patterns that are discussed below. The amorphous nature of the complexes involving the long chains might possibly be explained on the grounds that these chains cross-link between adjacent inorganic constituents, building up on increasingly complex reticulum as chain length increases. This cross-linking should become quite perceptible when the polyglycol includes more than 18 oxygen atoms, or at a molecular weight of about 800, and the X-ray diffraction data do indeed show that above this value the degree of crystallinity is markedly reduced.

It is the authors' belief that similar complexes with polyglycols lower than polyethylene glycol 200 are formed, and that ethylene glycol itself reacts in some fashion. These reaction products, according to this hypothesis, are not perceptible by the formation of precipitates and exist in solution in equilibrium with the hydrated barium silicotungstate. It seems probable that the greater number of hydroxyl groups in these complexes confers this property of water solubility, whereas the preponderance of ether linkages in the higher molecular weight polyglycols leads to water insolubility of the complexes containing them. Polyethylene glycols 200 and 300 seem to be intermediate in this respect. Under the conditions outlined above, the former precipitates only approximately one-tenth, and the latter approximately seven-tenths of the amount of barium silicotungstate precipitated by any of the higher members of the series. For this reason, blends containing any considerable amount of polyethylene glycol 200 could be detected by reason of the small amount of barium silicotungstate precipitated by them.

X-Ray Diffraction.—The solid complexes of barium silicotungstate with the polyglycols give X-ray diffraction patterns that are distinctly different from any of the reactants, namely, silicotungstic acid, the residue of ignited barium silicotungstate, or, in the case of the "Carbowax" compounds, the polyglycol

TABLE I.—ANALYSES OF THE BARIUM SILICOTUNGSTATE COMPLEXES OF SOME POLYGLYCOLS

	Nominal Mean Molecular Weight of Polyglycol					
	400	600	1000	1540	4000	6000
Sample taken, mg.	1.00	1.00	1.00	1.00	1.00	1.00
Complex precipitated, mg.	5.18	5.21	4.94	4.97	5.12	5.01
Ash of complex, mg.	4.15	4.15	3.91	3.92	4.03	3.89
Loss of weight on ignition, mg.	1.03	1.06	1.03	1.05	1.09	1.12
Water in complex, mg.[a]	0.03	0.06	0.03	0.05	0.09	0.12
Molar composition of complex						
Ash ($2BaO \cdot SiO_2 \cdot 12WO_3$)	1.00	1.00	1.00	1.00	1.00	1.00
Polyethylene glycol	1.90	1.26	0.81	0.52	0.20	0.13
PEG oxygen atoms	18.35	17.98	18.76	18.55	17.86	18.46
PEG ethoxyl groups	16.45	16.71	17.96	18.03	17.66	18.33
PEG ether oxygen atoms	14.56	15.45	17.15	17.51	17.47	18.19
Water	1.26	2.53	1.33	2.23	3.90	5.39

[a] Difference between line 4 and line 1.

itself. These patterns are strongly crystalline in the case of the lower molecular weights such as polyethylene glycols 400 and 600 (Fig. 2), but as the molecular weight of the polyglycol increases, there is a progressive decrease in the degree of crystallinity of the complex. In other words, the patterns show a progressive change in character as the molecular weight of the polyglycol component increases. This change consists of: (*a*) a decrease in the number of lines, (*b*) a decrease in the sharpness of the lines, and (*c*) a change in the position of certain lines.

Table II summarizes the observations made upon the diffraction photographs. In all of these patterns one important line may be discerned that defines what is referred to as a "long spacing." It is the authors' opinion that this "long spacing" represents the perpendicular distance between planes of symmetry having the maximum concentration of refracting atoms. Kraus (7) has given X-ray diffraction data upon barium silicotungstate having 24 molecules of water of crystallization, and from this the most prominent lattice dimension can be calculated. This value agrees closely with the mean value for the long spacings listed in Table II (exclusive of that for "Carbowax" compound 4000). It would appear, therefore, that these complexes are isomorphous with barium silicotungstate, each oxygen atom of the polyglycol replacing one molecule of water of crystallization. It is possible that the conditions under which the complex is precipitated limit such replacement to 18 out of the 24 water molecules, and the remaining 6 are partially driven off during the process of drying at 110°. In this connection it is of interest to note in Table I that the sum of the oxygen atoms and molecules of water for the "Carbowax" compound 6000 complex is almost 24, or 23.85 to be exact.

Ultrafiltration of Polyglycols.—The results obtained with the two types of cellophane membranes differed. The cellophane tubing exhibited a gradually decreasing permeability with increasing mean molecular weight, commencing early in the series with polyethylene glycol (PEG) 300. Under the conditions of this experiment the percentages of the respective compounds that passed through the membrane are as follows: PEG 200, 100%; PEG 300, 95%; PEG 400, 97%; PEG 600, 91%; "Carbowax" compound 1000, 80%; "Carbowax" compound 1540, 57%; "Carbowax" compound 4000, 43%; "Carbowax" compound 6000, 24%; and "Carbowax" compound 10,000, 20%. These percentages refer to only one sample of the polyglycol in question. Other samples may give slightly different results, but the trend in the series is preserved.

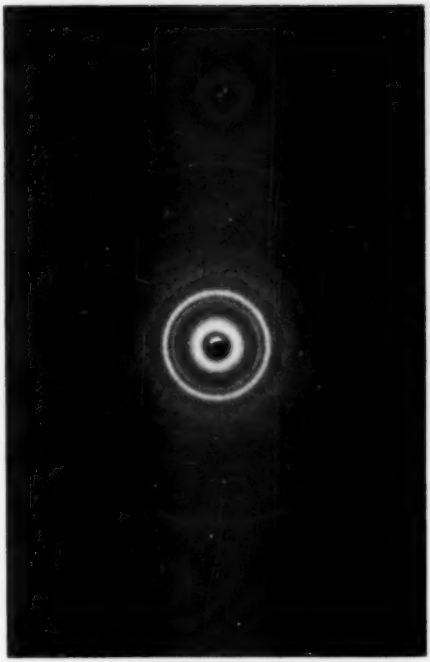

Fig. 2.—Monochromatic pinhole X-ray diffraction pattern of the barium silicotungstate complex of polyethylene glycol 400 (Flat Film No. F98).

TABLE II.—SUMMARY OF RESULTS OF X-RAY DIFFRACTION PHOTOGRAPHS OF THE BARIUM SILICOTUNGSTATE COMPLEXES OF VARIOUS POLYGLYCOLS

Sample	Flat Film No.	Powder Film No.	Long Spacing (Å.)	Error Limit (Å.)	Nature of Powder Pattern
PEG 400	F98	P583	26.8	±0.8	Fair pattern of about a dozen lines
PEG 600	F107	P616	26.6	±0.8	Very similar to the pattern of PEG 400, but slightly more diffuse and does not show the fainter lines
"Carbowax" compound 1000	F106	P615	27.0	±0.8	Pattern shows features transitional between those of the lower and higher molecular weight complexes. The most striking feature is the merging of two lines at 10.6 and 12.6 into one line at about 11.5 Å.
"Carbowax" compound 1540	F92	P588	25.3	±1.2	Pattern is similar to that of "Carbowax" compound 1000 but has fewer and somewhat more diffuse lines
"Carbowax" compound 4000	F99	P602	29.0	±1.2	Pattern resembles that of "Carbowax" compound 1540 but has still fewer lines and is somewhat more diffuse
"Carbowax" compound 6000	F100	P603	26.9	±0.8	Pattern is diffuse and shows only three lines

TABLE III.—THEORETICAL PERCENTAGE OF LOWER GLYCOLS IN SOME POLYGLYCOLS

Glycol	Per Cent by Weight in Polyethylene Glycol 200	300	400	600	"Carbowax" Compound 1000	1540
Ethylene glycol, $HO(CH_2CH_2O)H$	1.36	0.093	0.005	5×10^{-3}	4×10^{-9}	3×10^{-22}
Diethylene glycol, $HO(CH_2CH_2O)_2H$	7.26	0.86	0.069	1.1×10^{-2}	1×10^{-7}	2×10^{-20}
Triethylene glycol, $HO(CH_2CH_2O)_3H$	16.07	3.29	0.39	9.3×10^{-2}	2×10^{-6}	4×10^{-19}
Tetraethylene glycol, $HO(CH_2CH_2O)_4H$	21.70	7.67	1.34	4.9×10^{-2}	2×10^{-5}	5×10^{-18}
Pentaethylene glycol, $HO(CH_2CH_2O)_5H$	20.83	12.70	3.29	1.83×10^{-1}	1×10^{-4}	5×10^{-17}
Hexaethylene glycol, $HO(CH_2CH_2O)_6H$	15.46	16.26	6.24	5.29×10^{-1}	6×10^{-4}	4×10^{-16}

NOTE: The authors are indebted for these calculations to E. F. Hillenbrand, Jr., Works Control Laboratory, Carbide and Carbon Chemicals Corporation, South Charleston, W. Va.

The Du Pont sheet cellophane No. 600 PT exhibits the same type of graded response as the cellophane tubing with the significant exception that the diminution in permeability is observed only with compounds of mean molecular weight of 1540 and greater. Solutions of four different samples of "Carbowax" compound 1000, and six different samples of "Carbowax" compound 1540 were filtered through this particular type of membrane. The former, with one exception passed through the membrane completely, while at least 10% or more of the latter was retained. Under comparable conditions only 42% of a sample of "Carbowax" compound 6000 was filterable. It is concluded, therefore, that within certain limits the ultrafiltration technique is of value in detecting mixtures of polyglycols, one or more components of which are of high mean molecular weight.

Ethylene Glycol in Polyethylene Glycols 200 and 400.—In attempting to explain the variation in the amounts of barium silicotungstate precipitated by different batches of PEG 400, the possibility was considered that this circumstance was a result of a variable content of lower glycols, incompletely precipitated by the method. In order to obtain some independent evidence for this supposition, the percentage of substances oxidized by periodic acid, presumably ethylene glycol, was determined in quadruplicate in 14 batches of polyethylene glycol 400 which were available. These showed an average content of 0.117% ± 0.062 ethylene glycol, with extremes of 0.33% and 0.09%. Contrary to expectations the individual values did not correlate with the combining ratios with barium silicotungstate determined for those samples.

In the case of PEG 200, ethylene glycol was determined in duplicate in 12 samples. The average of the 24 analyses was 0.195% with a standard deviation of 0.024. Single determinations ranged from 0.16% to 0.23%.

It is of interest to compare these values for ethylene glycol with those that may be calculated for these products from Flory's considerations, since this is the only individual instance in which the theory can specifically be tested. Reference to the data in Table III will show that the actual amount of ethylene glycol present is low compared to the theoretical in the case of PEG 200, but high for PEG 400. It must be pointed out, however, that there is no assurance that the conditions required for the calculation have been fulfilled in the manufacture of any of the individual products tested. Since these particular analyses were performed a special grade of PEG 400 has become commercially available in which the amount of ethylene and diethylene glycol combined is held to a low level.

SUMMARY AND CONCLUSIONS

Certain chemical and physical data are furnished on a series of polyglycols commencing with polyethylene glycol 200 and ending with "Carbowax" compound 10,000. Some of these compounds are distinguished by their precipitability by barium silicotungstate, and most of them exhibit an inverse relationship between ultrafilterability and mean molecular weight. Chemical and X-ray diffraction studies of the polyglycol-barium silicotungstate complex are presented, and a hypothesis as to its structure is advanced. Information is given on the presence of ethylene glycol in two polyglycols, and a comparison is made of these values with those calculated according to Flory's theory.

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The Toxicology of the Polyethylene Glycols*

By HENRY F. SMYTH, JR., CHARLES P. CARPENTER, and CARROL S. WEIL

Information developed during recent years upon the toxicological actions of the fluid and solid polyethylene glycols is summarized. This information has been re-interpreted and supplemented. Evidence presented in this report confirms the earlier work indicating that acute oral toxicity, dermal toxicity, and irritating properties of the polyethylene glycols are very low. The safe rat dose of "Carbowax" 1500 is now known to be substantially greater than originally reported, and of "Carbowax" 4000, slightly greater.

TOPICAL applications of polyethylene glycols are increasing rapidly and preparations involving internal dosage are receiving attention. Information from this laboratory upon the toxicological and physiological actions of the fluid and solid polyethylene glycols has appeared in several journals over a period of years (1–8). The present article and its companions (11, 12) have been prepared to collect and summarize all of these data in one place and to add supplemental facts not previously published.

Polyethylene glycols 200, 300, 400, and 600 are fluids, "Carbowax" compounds 1000, 1540, 4000, 6000, and 10,000 are solids of increasing firmness, and "Carbowax" compound 1500 is a solid consisting of a blend of "Carbowax" compound 1540 and polyethylene glycol 300 in substantially equal weights. The number gives the approximate average molecular weight of the polyethylene glycols in the particular product. Reference will be made to single compounds in abbreviated form, for example PEG 400 and "Carbowax" 4000.

The general chemical formula representing any member of the series may be written in a variety of ways. One form is shown below:

$$HOCH_2CH_2(OCH_2CH_2)_nOH$$

Here n is one less than the number of ethylene oxide units employed in building the molecule and diethylene glycol is the lowest member of the series. At one point in this paper it is convenient to consider ethylene glycol to be the lowest member.

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ACUTE ORAL TOXICITY

Acute oral toxicity was estimated upon male albino rats weighing 90 to 120 Gm. when dosed. Rats of the Wistar strain were used through 1945, while since that time Sherman strain animals from Rockland Farms have been the subjects. The two strains have shown identical resistance to some materials. Guinea pigs were of mixed sexes weighing from 200 to 300 Gm., from a variety of sources. Rabbits were male albino New Zealand giants weighing 2 to 3 Kg. Both Purina and Rockland Farms laboratory diets have been fed.

Compounds were administered by stomach tube as approximately 50% solutions in water, 1 ml. containing 0.50 Gm., except that PEG 200, 300, and 400 were administered undiluted to rabbits. The animals were not fasted before dosing. All species were observed for fourteen days following the dose.

Table I lists the oral LD_{50} values for all samples of polyethylene glycols tested to the present, identified by the year in which they were manufactured. About half of the values were previously published. It is evident from the data that the LD_{50} values on different samples of one product varied in a numerical sense, but in general the variation is no greater than the range of uncertainty in the method of determination.

There is shown a clear tendency for a slight decrease in acute toxicity with large increases in molecular weight. Data upon intestinal absorption (6) make it illogical to believe that the high molecular weight polyglycol molecules are absorbed from the digestive tract in sufficient numbers to kill animals in acute toxicity tests. It seems probable that some more diffusible molecules present in the commercial products are responsible. So far no method has successfully isolated, removed, or identified the hypothetical product.

Rats which are victims of large single doses of polyethylene glycols of any molecular weight yet tested, die with renal lesions similar to those resulting from smaller amounts of ethylene and diethylene glycols. Cloudy swelling of liver parenchyma is also found. With "Carbowax" 1500, 1540, and 4000 there are no victims at dosages of 32 Gm./Kg. or less. After dosages of 20 Gm./Kg. or less of these three compounds, rats have microscopically normal livers and kidneys at two, seven, and fourteen days. After higher dosages a portion of the survivors have renal and hepatic cloudy swelling, most prominent at seven days, and the remainder of the rats are normal (2).

Among rabbits given 50 Gm./Kg. of "Carbowax" 4000 the blood urea level of victims rose to about four times normal, and that of survivors either rose to about twice normal and then fell, or more often was not affected (2).

TABLE I.—SINGLE ORAL DOSE TOXICITY OF PRODUCTS IN THE POLYETHYLENE GLYCOL SERIES

Compound[a]	Year Produced[b]	Male White Rat LD_{50}, Gm./Kg.	Male White Rat Fiducial Range[c]	Male White Rat Reference	Other Species	Other Species LD_{50}, Gm./Kg.	Other Species Fiducial Range[c]	Other Species Reference
Ethylene glycol[e]	1938	8.5	7.3–10.0	(1)	Rabbit	13.6	12.1–15.2	(1)
					Guinea pig	6.6	5.1– 8.6	
	1939*	13.7	11.0–17.1					
Diethylene glycol[b]	1937A	20.8	19.5–22.1	(1)	Guinea pig	13.2	11.6–15.0	(1)
	1937B	32.4	30.2–34.8	(1)				
	1939*	25.8	24.2–27.6	(1)				
Triethylene glycol	1938	22.1	19.4–25.1	(1)	Guinea pig	14.7	13.8–15.7	(1)
	1939*	25	RF					
PEG 200 (Tetra)	1938	32.8	28.2–36.3	(1)				
	1940	36.7	33.2–40.5					
	1944	28.9	25.7–32.5	(3)	Rabbit	19.9	17.0–23.4	(3)
	1948	34.0	30.7–37.6					
PEG 300 (Hexa)	1939	31.6	28.6–35.0	(1)	Guinea pig	19.6	14.5–26.4	(1)
	1940A	27.5	25.4–29.8					
	1940B	38.8	36.7–41.1					
	1944	31.7	28.4–35.5	(3)	Rabbit	20.7	18.9–22.7	(3)
	1947	45.6	43.3–48.2		Rabbit	17.3	14.9–20.0	
	1949	38.9	35.9–42.2					
PEG 400 (Nona)	1939	30.2	27.8–32.4		Guinea pig	15.7	9.7–28.3	
	1940	37.4	34.2–41.0	(1)				
	1944	32.8	28.8–37.4	(3)	Rabbit	26.8	24.3–29.6	
	1948	43.6	41.7–45.6					
"Carbowax" 1000	1945	42.	RF		Guinea pig	22.5	18.9–26.8	
"Carbowax" 1500	1940	44.2	44.2–44.2	(1)	Rabbit	28.9	26.5–31.4	
					Guinea pig	28.9	25.5–34.7	(2)
"Carbowax" 1540	1940	51.2	45.7–57.4	(1)				
"Carbowax" 4000	1940	>50	RF	(1)	Rabbit	76	RF	
					Guinea pig	>50	RF	
	1940*	>50	RF		Guinea pig	50.9	39.1–66.1	
	1946[d]	59	RF					
"Carbowax" 6000	1941	>50	RF		Guinea pig	>50	RF	
"Carbowax" 10,000	1949	50	RF					

[a] PEG stands for polyethylene glycol of the average molecular weight shown. The parentheses give the average number of ethoxy groups.

[b] A and B signify two samples made the same year. An asterisk * shows a specially purified sample, molecularly distilled if a fluid or recrystallized if a solid.

[c] The range of ± 1.96 standard deviations. "RF" signifies that too few animals were used to allow calculation of a fiducial range because of inadequate sample or low mortality at maximum possible dosage.

[d] Data obtained from divided doses: Rats, LD_{50} 96 Gm./Kg. RF; 1/4 of dose every three hours. Rabbits, LD_{50} 84.9 (78.4 to 91.9) Gm./Kg.; 1/4 of dose every two hours.

[e] Ethylene glycol is not actually a polyglycol.

SUBACUTE AND CHRONIC ORAL TOXICITY

In 1947 we published (7) the results of ninety-day oral doses to rats for the 1944 production of PEG 200, 300, and 400. A concentration of 16% in the drinking water (11 to 20 Gm./Kg. per day) reduced fluid intake and killed two-thirds of the animals. Victims had necrosis of renal tubular epithelium but survivors of the same dosage level had, for the most part, normal kidneys. There was no effect from 4% levels in the drinking water which were equivalent to about 5 Gm./Kg. per day.

A similar subacute study was performed upon 1948 production of the three polyglycols. PEG 200 at 8% in the water killed all rats with severe kidney pathology within fifteen days, and at 4% (8.0 Gm./Kg. per day) 2 of 9 rats died after eighty days with liver and kidney pathology but the 7 survivors were normal. PEG 300 at 16% killed all within seven days, 8% killed 2 of 10 in sixty days, 4% (7.2 Gm./Kg. per day) had no effect. PEG 400 at 16% killed all within thirteen days, 8% and 4% (20.0 and 7.5 Gm./Kg. per day) had no effect except that kidneys were somewhat lighter in weight than those of control rats.

Among these three polyethylene glycols, toxicity to rats in repeated doses decreases with increasing molecular weight.

During a ninety-day dosage series rats drank 16% solutions of 1941 production of "Carbowax" 1500 and 4000 as freely as control rats drank water, and none died (2). Growth was equal to that of the control rats. As a dosage of 4.05 Gm. or more of "Carbowax" 1500 or 0.08 Gm. or more of "Carbowax" 4000 per Kg. per day a few kidneys had cloudy swelling. At 0.23 Gm. or more of the latter compound per Kg. per day, testicular degeneration was occasionally found. It was concluded that 1.50 Gm. of "Carbowax" 1500 or 0.04 Gm. of "Carbowax" 4000 per Kg. per day was without effect on rats during the ninety-day experiment.

Doses of 1941 production of "Carbowax" 4000 were given by stomach tube to rabbits five days a week for five weeks, 20 Gm./Kg. per day being the greatest dosage. All survived, blood urea remained normal, and renal pathology was absent. The greatest dosage prevented normal growth. Single doses of 50 Gm./Kg. markedly elevated blood urea of some rabbits and did not affect blood urea of others (2).

An additional ninety-day dosage series was conducted on 1947 production of "Carbowax" 4000. In this hitherto unpublished experiment the highest dosage fed, 2.5% in the diet, equivalent to about 1.6 Gm./Kg. per day, did not reduce growth or produce any renal or other pathology. This finding is in marked conflict with results on 1941 production and indicates that current output is less toxic chronically than that of earlier years. At the same time 1947 production of "Carbowax" 1540 and later 1949 production of "Carbowax" 10,000 were studied with the same result; 2.5% in the diet was without effect on the rats as evidenced by food eaten, weight gain, weight and pathology of liver and kidney.

In 1947 we published the results of two-year oral doses to rats of 1941 production of "Carbowax" 1500 and 4000 (7). In that study of low dosages, life span, fertility, micropathology, and blood cytology were normal. The only effect was a 9% retardation of growth in body weight at higher levels, 0.059 Gm. of "Carbowax" 1500 and 0.017 Gm. of "Carbowax" 4000 per Kg. per day being the greatest intakes which allowed growth equal to that of control rats.

In the two-year study only one rat of the first filial generation survived to one year of age in the group receiving no dose whatever. It seemed important to consider the generations separately and one rat in a group was too few to justify a comparison. Accordingly a synthetic control group was formed by combining the observations on rats receiving no doses with those receiving the least dosage of each of the two "Carbowax" compounds. It was in comparison with the growth of the synthetic control group that growth of rats receiving greater dosages was retarded. We now believe that the use of this synthetic control group was unwarranted and misleading. The data of the experiment have been reconsidered by comparing the weights of dosed rats with only undosed controls and the results of the comparison are discussed below.

Table II shows the differences between average weights of dosed and control rats, weight being considered from several points of view. A footnote in the table marks entries where the difference is statistically significant by the familiar "t" test, using 0.05 as the fiducial limit. It is only in the maximum weight attained by females receiving the three lower dosages of "Carbowax" 1500 that differences from the controls are statistically valid, and the difference at the greatest dosage is not valid. In a study involving response to graded dosages, judgment dictates that we reject a statistically significant adverse difference from the control at some intermediate dosage if it is not confirmed by a similarly significant adverse difference at a higher dosage. We explain the occurrence of such "skips" by the hypothesis that here we may have the 1 in 20 occurrence of random variation which is not eliminated by the "t" test with a fiducial limit of 0.05. Usually such "skips" vanish if we change our fiducial limit to 0.01. In the study of this weight data 200 "t" tests were performed, only 40 being tabulated in Table II. Nine of them are classified as "skips" not confirmed by similar adverse results at higher dosages or by similar stimulation at lower dosages. The ratio is 0.045, almost precisely the proportion of excessive random variations which our fiducial limit of 0.05 would be expected not to exclude. We regard this frequency of "skips" as justification for following judgment in disregarding them. Accordingly the suggestion from maximum weight attained by females is disregarded.

In most instances the weight differences given in Table II suggest trends of effect associated with the dosage. Negative correlation coefficients between dosage and weight are shown in 9 of the 10 cases tested. Three coefficients are large enough to be statistically significant. The one positive coefficient is also significant, but we would not wish to claim that this proves the unlikely hypothesis that the more "Carbowax" 1500 a female rat eats the heavier the rat will be. There is no doubt that "Carbowax" 4000 had some influence on the weight of the rats, and at some dosage higher than those fed this influence would produce rats growing significantly less than the control rats. The correlation coefficients are low and the corresponding "p" values are not far from the fiducial limit. There is no basis, we feel, for regarding the correlation as revealing injury from "Carbowax" compounds which was not revealed by the "t" test on single dosage levels.

We believe that the study of the results of feeding graded dosages by the calculation of correlation coefficients does not add materially to the value of toxicological experiments. It will confirm and evaluate trends evident on simple inspection of the data. But quantitative toxicology is concerned with determining the maximum harmless dosage. It is a self-evident fact that some high dosage will injure

TABLE II.—WEIGHT OF RATS DURING TWO-YEAR ORAL DOSES OF "CARBOWAX" COMPOUNDS[a]

	Compound Administered and Percentage in Drinking Water									
	"Carbowax" 1500					"Carbowax" 4000				
Criterion Examined	2.0	0.4	0.08	0.02	Correlation Coefficient	0.08	0.02	0.005	0.00125	Correlation Coefficient
Wt. at 380 days of age	−21	−22	+17	+22	−0.27	−24	− 2	+13	+76	−0.37[b]
Wt. gain 40 to 380 days of age	+18	−15	+22	+23	−0.30	−17	− 8	+ 7	+28	−0.32
Av. wt. from 40 to 380 days of age	−22	−22	+ 2	+12	−0.29	−23	+ 4	+ 9	+19	−0.38[b]
Max. wt. attained by males	−31	−18	+14	+11	−0.30	−28	−16	+17	+53	−0.39[b]
Max. wt. attained by females	−36	−73[b]	−59[b]	−76[b]	+0.23[b]	−24	−50	−38	+15	−0.10

[a] Tabular entries give difference between treated and untreated rats in the particular criterion examined. Under "Correlation Coefficient" is shown the correlation between dosage and weight for all groups receiving the same compound.
[b] Statistical evaluation shows difference from control is beyond fiducial limits of $p > 0.05$.

and we feel that the confirmation of a trend by a correlation coefficient is redundant. No matter how good the correlation in a series of dosages, it does not alter the importance of the fact that one particular dosage did or did not cause a difference from the controls greater than would be expected from random causes.

We have now denied our published statement (7) that 0.27 Gm./Kg. per day of "Carbowax" 1500 and 0.062 Gm./Kg. per day of "Carbowax" 4000 over a two-year period reduced the growth of rats, and we conclude that we found no injury from the highest dosages fed, 1.69 and 0.062 Gm./Kg. per day, respectively. There are clear indications that at some level, possibly not greatly above these dosages, a decrease in growth would have resulted. It remains to inquire whether the altered conclusions materially change the picture of the chronic toxicity of the two "Carbowax" compounds.

The 1942 paper (2) gave results of ninety-day oral doses to rats. "Carbowax" 1500 at 4.05 Gm./Kg. per day produced minor reversible kidney pathology in some rats but at 1.50 and lower kidneys were normal. The new conclusions on two-year doses hold that 1.69 Gm./Kg. per day did not injure rats. The two figures are mutually confirmatory. The 1947 paper (7) suggested 0.069 Gm./Kg. per day as the safe rat dose. The newly defined safe rat dose is 20 times as great. This is sufficiently different materially to change judgments of the safety of particular proposed human applications of "Carbowax" 1500.

In ninety-day doses "Carbowax" 4000 was reported (2) to cause minor kidney pathology in all rats at 0.23 Gm./Kg. per day and in some rats at 0.08. Current interpretation of the two-year doses is that 0.062 Gm./Kg. per day did not injure rats, which is consistent with the ninety-day conclusions. It therefore appears that the safe rat dose of "Carbowax" 4000 is at least 0.06 Gm./Kg. per day, about three times the previously published value (7). It appears doubtful if this change will materially alter judgments of the safety of proposed applications of "Carbowax" 4000.

A complicating factor is the indication, mentioned a few paragraphs above, that 1947 production of "Carbowax" 4000 has less chronic toxicity than the 1941 material studied in most detail. The results on 1949 "Carbowax" 10,000 tend to confirm indications of low toxicity and prove there is no trend to greater subacute toxicity with greater molecular weight. Thus considerable doubt is cast upon the results of two-year doses of "Carbowax" compounds which used 1941 products. Improvement in product is not unusual as chemicals grow into larger production.

PARENTERAL TOXICITY

The LD_{50} values of several "Carbowax" compounds by intraperitoneal injection in rats were published in 1947 (7). Since that time a second batch of "Carbowax" 4000 was found to check the order of magnitude of the toxicity of the first batch and both "Carbowax" 10,000 and PEG 300 have been tested in this way. Fifty per cent solutions in water were sterilized by steam pressure and were injected intraperitoneally into male rats weighing 90 to 120 Gm. Survivors were observed for fourteen days, although 80% of the deaths occurred within thirty hours of injection. After fourteen days it was shown analytically that no "Carbowax" 4000 remained in the peritoneal cavity. The values are listed in Table III.

TABLE III.—PARENTERAL TOXICITY

Compound	Year Produced	Rat Intraperitoneal LD_{50}, Gm./Kg.
PEG 300	1946	17.0 (15.6–18.5)
"Carbowax" 1000	1945	15.6 (12.8–18.9)
"Carbowax" 1500	1940	17.7 (15.3–20.5)
"Carbowax" 1540	1940	15.4 (13.7–17.4)
"Carbowax" 4000	1940	11.6 (9.6–13.9)
"Carbowax" 4000	1946	13.0 (10.1–16.8)
"Carbowax" 6000	1941	6.8 (3.6–13.0)
"Carbowax" 10,000	1949	12.6 (3.5–45.6)

All the compounds manifested a low order of intraperitoneal toxicity. The value for "Carbowax" 10,000 has such a high uncertainty that it is of little use. It is of interest that by this route toxicity increases with increasing molecular weight if we do not consider the doubtful final entry, the reverse of the relation found with oral doses. This observation suggests that the low order of oral toxicity is partly due to poor absorption of larger molecules.

In 1941 we estimated rat intravenous LD_{50} values for "Carbowax" 1500 and 4000. Injections of 20% solutions in saline were made rapidly, into the tail vein. Rats died promptly, and LD_{50} values were close to 8.5 and 7.5 Gm./Kg., respectively. Control rats given saline alone proved that large volumes were not a sufficient insult to account for fatalities. The results were never published because precipitate injection was judged to be a poor criterion of toxicity.

Later work (7) showed that *in vitro* 10% or more concentrated solutions of "Carbowax" compounds will clump the cellular elements of rabbit, dog, or human blood. Microscopically clumps were seen in the blood of rabbits that died from 20% solutions given in the ear vein. Accordingly, it was concluded that the rapid intravenous injections of 1941 had caused rats to die of thrombosis.

All compounds listed in Table I except "Carbowax" 10,000 were given by ear vein to two male rabbits each at a level of 10 Gm./Kg. (7). Injection was made by machine at the rate of 2.5 ml. of 5% sterile solution per minute. The volume injected was approximately 500 ml., an amount greater than the total blood volume of the animal. All rabbits survived fourteen days in good health, most of them gained weight, and the only microscopic evidence of effect found was cloudy swelling of the renal tubular epithelium in one rabbit each of those receiving "Carbowax" 4000 and 6000. Similar effects are regularly seen from large intravenous injections of any innocuous fluid. During the injections blood levels of 5 to 10 mg. "Carbowax" per ml. were obtained and urine concentrations as great as 90 mg./ml. were found.

The same compounds were given to groups of five rabbits by aseptic injection into the ear vein at a dosage of 1 Gm. per rabbit, repeated daily six

times a week for five weeks (7). The dosage averaged 350 mg./Kg. per day. Three out of a total of 45 animals died from the doses, one each from PEG 400, and "Carbowax" 4000 and 6000. Six other rabbits died from infections judged to be irrelevant to the injections. Twenty-nine of the 34 that did not die gained weight during the experiment. Blood urea levels were not elevated except in the three which died from the doses. No pathology more severe than cloudy swelling of renal tubular epithelium and hepatic parenchyma was seen, and these effects were evident in only 9 of the 45 rabbits, distributed among all the eight compounds tested.

It is evident that the intravenous toxicity of dilute solutions of polyethylene glycols is low.

SKIN PENETRATION TOXICITY

The doses required to kill animals by penetration through the skin are so large that estimates of acute toxicity by this route are impossible. We have already reported (3) that in the twenty-four-hour cuff test 20 ml./Kg. of PEG 200 killed one of six rabbits within fourteen days while PEG 300 and 400 killed none of six. A 1948 sample of PEG 300 at this dosage killed one of ten rabbits. We are unable to keep larger single doses in contact with the rabbit skin by this method.

Subacute inunction applications of PEG 200, 300, and 400 were made upon rabbits five days a week for eighteen weeks (3) at a level of 2 ml./Kg. per day without fatalities. Body weight, blood urea, and icteric index of treated rabbits did not differ from those of control animals of the same age. We have applied "Carbowax" 1500 and 4000 to rabbits only by poulticed applications (2). Doses of 10 Gm./Kg. held on the skin for five days a week over a period of thirteen weeks resulted in no fatality and blood urea values were not affected. White petrolatum was similarly applied to control animals. "Carbowax" 4000 produced less skin irritation than did petrolatum and "Carbowax" 1500 caused none whatever.

Luduena and associates (9) applied PEG 200 and 400 and "Carbowax" 1500 and 4000 to mature mongrel rabbits by inunction six days a week for five weeks. They obtained increases in blood urea, kidney injury, and fatalities among animals eating an incomplete diet that consisted of rolled barley, at dosage levels of 1 or 2 Gm./Kg., and considerably less injury upon a more complete diet of "Purina Rabbit Chow Complete Ration." There were no control rabbits to allow judgment as to mortality or kidney effects of the deficient diet alone and accordingly the significance of their observations cannot be evaluated.

PRIMARY IRRITATION

Primary irritation was not evident in the applications to rabbit skin discussed above. On the mucous membrane of the rabbit eyelid 20% solutions of PEG 200, 300, and 400 gave capillary injection closely comparable to that from 10% glycerin U. S. P. (3). "Carbowax" 4000 in 10% solution was equivalent to 2% glycerin on this site (2). There was no corneal injury from any of the compounds discussed here when tested on the rabbit eye (4).

SKIN SENSITIZATION

In 1946 intracutaneous guinea pig sensitization tests were performed, following the technique described by Draize (10) except that 8 instead of 10 injections were given and three weeks were allowed for development of sensitivity instead of two. There were 13 to 16 guinea pigs used for each compound. No sensitizations were obtained with PEG 200 and 300. PEG 400 caused some degree of sensitization response in 62% of the animals, the worst reaction being an area of edema less than 1 mm. in diameter. "Carbowax" 1500 and 4000 caused 77% and 74% sensitization responses, respectively, the worst reaction in each case being necrosis less than 1 mm. in diameter. It has been our experience that this guinea-pig intracutaneous sensitization test agrees with the results of human dermal contact in about one-fourth of the instances where we can compare the two, the intracutaneous test producing response with all human sensitizers and with twice as many compounds which very seldom sensitize any human skin.

In 1949 human patch tests were performed for us by a dermatologist on current production of PEG 400 and "Carbowax" 4000. No reactions of any kind were obtained on the group of 100 male and 100 female subjects. We reported earlier (2) that 1940 production of "Carbowax" 1500 and 4000 had produced 3 and 4 sensitization reactions, respectively, consisting of faint erythema, when we patch-tested 100 male subjects. A similar frequency of response was elicited by lanolin, petrolatum, simple cerate, and simple ointment. At about the same time PEG 200, 300, and 400 were applied to 23 subjects and similar mild sensitization reactions were obtained on 22, 9, and 4% (3).

DISCUSSION

"Carbowax" compounds of molecular weights above 1000 have been found not to be absorbed from the gastrointestinal tract of the rat (6). The analytical means used in arriving at this conclusion are capable of detecting only polyethylene glycol molecules and cannot give information about the absorption of hypothetical by-products formed during manufacture. Two-year oral doses to rats of 1941 "Carbowax" 4000 indicate a safe dose unreasonably small for a compound of extremely low acute toxicity which is not absorbed from the digestive tract. On this basis we feel that there was a diffusible by-product present in the 1941 product, but there is no proof of the hypothesis.

Ninety-day oral doses to rats of 1947 production of "Carbowax" 4000 show a much lower toxicity than did ninety-day doses of the 1941 product. This observation leads us to believe that improvement in manufacture may have reduced the amount of the hypothetical by-product until it is of little significance. No long-time feeding tests which might confirm the belief have been performed, but ninety-day doses of "Carbowax" 10,000 lend support.

It is clear that the acute oral and dermal toxicity and irritating powers of polyethylene glycols are very low. Parenteral toxicity is of more concern because of the power of some of the compounds in high concentration to cause clumping of cellular elements of the blood.

SUMMARY

Previously published data on the toxicity of polyethylene glycols are discussed and slightly extended. A re-interpretation of two-year doses of 1941 production results in materially increasing the safe rat dose of "Carbowax" 1500 and slightly increasing that of "Carbowax" 4000. The new values are, respectively, 1.5 and 0.06 Gm./Kg. per day, but subacute doses of current production indicate without defining considerably greater safe rat doses.

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Comparison of Analgetic Activity of 67 Arylalkamines with That of Morphine and Meperidine by the Mouse Hot Plate Method*,†

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A total of 67 arylalkamines have been compared with morphine and meperidine activity by the hot plate method using mice. Out of the total number of arylalkamines investigated only iso-1,2-diphenylethanolamine hydrochloride produced a fair degree of depression to pain. Slight action was demonstrable with five of the others.

PRIOR to the reports of Dodds, Lawson, and Williams (1–3) concerning the analgesia produced by α,β-diphenylethylamine and structurally related compounds, the synthesis of a series of α,β-diphenylamines was already under way (4), for the purpose of testing these compounds as antispasmodic agents. It now seemed desirable to continue the study of the pharmacology of these and chemically related compounds by screening them for possible analgetic activity, which tests are described in detail in this paper. The method is essentially that of Woolfe and MacDonald (5).

The purposes of our study were to secure some idea of the dose which would prove fatal, to determine the major toxic symptoms, and to ascertain whether or not any analgesia could be produced by doses well below those in the fatal range.

METHOD

Male albino Swiss mice of uniform stock,[1] weighing 24–35 Gm. were used unless otherwise specified; no mouse was used more than once. The drugs were injected in aqueous solution under the skin of the back just anterior to the tail. We usually started with 55.4 mg./Kg. of body weight, increasing the dose in several steps of 100% each time so as to ascertain quickly the toxic and fatal dosage levels. In subsequent testing we tried various dosage levels in between some of those first employed or, in some cases, below the 55.4 mg./Kg. dose, using 15% dosage increases. No special provision was made for control of room temperature which varied between 22° and 28°, largely according to season.

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‡ The authors are indebted to George A. Breon and Co. for supplying the compounds used in this study. Those with code numbers were prepared by this company.

[1] Fifteen mice on B-41 and all on B-42, B-44, B-45, B-46, B-47, B-48, B-49 B-50, and B-51 were supplied by a different dealer.

The evaluation of the analgetic action of these amines was made by using heat as the stimulus. The apparatus was similar to that used by Woolfe and MacDonald (5). A 15-cm. Fisher "Autemp" electric heating plate was used with a 170-ohm slide wire rheostat in series. A hollow celluloid cylinder enclosed the plate so that the mouse could be put on the plate and kept there until removed by hand. A copper tube was built into the top of the plate for insertion of a thermometer; all tests were made at a plate temperature of 60°. A stop watch was started when a mouse was gently dropped on the plate and stopped when it kicked its hind legs, started to lick the hind paws, or attempted to jump out of the restraining cylinder. Because normal mice often sit up and groom their front paws, which grooming might be mistaken for action of discomfort, we used only the movements of the hind limbs as our criterion. Several control readings were made before injecting any drug. To avoid any permanent damage to the feet, a mouse which did not respond to the heat stimulus in thirty seconds was removed. Each mouse was tested every ten minutes until either we were certain that the compound in question possessed no analgetic properties, or, if analgetic action appeared, until it disappeared, which was usually within ninety to one hundred and twenty minutes. The experimental results have been summarized in Table I.

So as to show the various aspects of the degrees of analgetic actions more clearly we have tabulated the results obtained with three selected arylalkamines for comparison with those exhibited by morphine and meperidine in Table II.

DISCUSSION

The highly abbreviated descriptions of the toxic symptoms shown by the drugs studied here seem sufficient in this type of screening tests. As may be seen from the results, the most effective of the new drugs, iso-1,2-diphenylethanolamine, the one previously reported active by Woolfe and MacDonald, was not on a par in analgetic activity with either meperidine, nor the still more potent morphine (see Table II). Apparently, our stock of mice was more sensitive to meperidine than that studied by Woolfe and MacDonald (5), though not to antipyrine. None of the remaining 66 arylalkamines approached closely the analgetic potency of iso-1,2-diphenylethanolamine. An extensive series of aralkylamines was studied in the cat by Fellows and Ullyot (6). The reader is referred to their report. From the results of tests upon papaverine, antipyrine, aminopyrine, and sodium salicylate, respectively, it will be noted that none of these produced any appreciable analgetic effect as judged by this method.

The time limit of thirty seconds is arbitrarily chosen to indicate a significant degree of analgetic potency and partly to prevent injury to the feet of the mice. By the method illustrated in Table II, one may readily compare new drugs with meperidine, and morphine by the hot plate technique. In the present case, one may also note that the more potent drugs reach maximum activity more rapidly than the less powerful ones. For quantitative comparison one may, by choosing the optimum analgetic dose and studying a sufficient number of mice, calculate the average number of times the mice remained on

TABLE I.—FORMULAS, COMPARATIVE TOXICITY, AND ANALGETIC EFFECTIVENESS OF 67 ARYLALKAMINES

Code No.	Ar	Ar—CH(OH)—CH(Y·HCl)—Ar′ Ar′	Y	Dose Range, Mg./Kg. and No. of Mice ( )	Lowest Dose Fatal to Any Mouse	Highest Dose Survived by Any Mouse	Toxic Symptoms	Pain Depression	References
B-5	Phenyl	Phenyl	Amino(normal)	55–509 (9)[a]	336	222	Dorsiflexion of tail; clonic convulsions; delayed death	None	(7)
B-7	Phenyl	Phenyl	Amino(iso)	55–509 (46)	443	336	Dorsiflexion of tail; tremors; violent convulsions; eschars at injection sites	Fair	(8)
B-22	Phenyl	Phenyl	Amino(*d*-iso)	48–886 (8)	443	222	Violent convulsions	None	(9)
B-35	Phenyl	Phenyl	Amino(*l*-iso)	55–886 (9)	222	146	Tremors and violent convulsions	None	
B-1	*p*-Methoxyphenyl	Phenyl	Amino(normal)	55–886 (13)	569	443	Tremors and violent convulsions	None	(10)
B-2	*p*-Methoxyphenyl	*p*-Methoxyphenyl	Amino(iso)	55–1344 (17)	772	672	Dorsiflexion of tail; tremors and violent convulsions	None	(11)
B-9	2,3-Dimethoxyphenyl	Phenyl	Amino(iso)	111–585 (10)	336	292	Slight depression with lower, increased reflexes and violent convulsions with higher doses	None	(12)
B-12	3,4-Dimethoxyphenyl	3,4-Dimethoxyphenyl	Amino(iso)	111–1344 (11)	1344	1170	Severe twitchings, dyspnea, and rolling on the side	None	(12)

(*Continued on p. 356*)

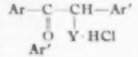
TABLE I.—FORMULAS, COMPARATIVE TOXICITY, AND ANALGETIC EFFECTIVENESS OF 67 ARYLALKAMINES (*Continued*)

Code No.	Ar	Ar'	Y	Dose Range, Mg./Kg. and No. of Mice ( )	Lowest Dose Fatal to Any Mouse	Highest Dose Survived by Any Mouse	Toxic Symptoms	Pain Depression	References
B-51	Phenyl	Phenyl	Methylamino	55–886 (5)	886	443	Tremors and clonic convulsions	None	(13)
B-47	Phenyl	Phenyl	N-Piperidyl	37–443 (10)	...	443	Excitement with lower, depression with higher doses	None	(12)
B-66	*p*-Methoxyphenyl	*p*-Methoxyphenyl	Benzylamino	55–1773 (6)	...	1773	Mice quiet or normal	None	(12)
B-72	*p*-Methoxyphenyl	Cyclohexyl	N-Piperidyl	48–1773 (8)	...	1773	Tranquility with lower doses; tremors with 1773	None	(12)

$Ar—CH_2—CH(NHR'\cdot HCl)—Ar'$

	Ar	Ar'	R'						
B-6	Phenyl	Phenyl	Hydrogen	73–509 (23)	222	256	Violent convulsions; dorsiflexion of tail	None	(1)
B-8	*p*-Hydroxyphenyl	*p*-Hydroxyphenyl	Hydrogen	55–1773 (20)[b]	772	886	Violent convulsions with high doses; quiet on low doses	None	(12)
B-3	*p*-Methoxyphenyl	*p*-Methoxyphenyl	Hydrogen	55–443 (15)	222	193	Dorsiflexion of tail; tremors and violent convulsions	None	(14)
B-63	Phenyl	*p*-Methoxyphenyl	Hydrogen	24–443 (8)	111	55	Tremors and violent convulsions; bloody nose	None	(15)
B-40	Phenyl	Phenyl	Methyl	55–886 (9)	443	222	Circus movement; violent convulsions	Very slight	(16)
B-30	Phenyl	Phenyl	Ethyl	32–886 (11)	585	443	Tremors and clonic convulsions	None	(14)
B-56	Phenyl	Phenyl	Allyl	55–886 (5)	443	222	Tremors and violent convulsions; delayed death	None	(16)
B-15	Phenyl	Phenyl	2-Hydroxyethyl	55–772 (8)	672	443	Tremors and violent convulsions	None	(14)
B-20	Phenyl	Phenyl	2-(4-Morpholino)ethyl	55–886 (10)	443	222	Clonic convulsions and opisthotonus with higher, depression and tremors with lower doses	Slight	(14)
B-32	*p*-Hydroxyphenyl	*p*-Hydroxyphenyl	Methyl(hydrobromide)	55–886 (5)	886	443	Convulsive tremors with high dosage	None	(12)
B-29	*p*-Hydroxyphenyl	*p*-Hydroxyphenyl	Ethyl(hydrobromide)	55–886 (5)	886	443	None listed	None	(12)
B-33	*p*-Methoxyphenyl	*p*-Methoxyphenyl	Methyl	55–886 (37)	84	111	Dorsiflexion of tail; clonic convulsions; tranquility with low doses	Slight	(14)
B-34	*p*-Methoxyphenyl	*p*-Methoxyphenyl	Ethyl	55–886 (5)	111	55	Mild bouncing convulsions	None	(14)
B-86	*p*-Methoxyphenyl	*p*-Methoxyphenyl	2-Hydroxyethyl	16–886 (9)	443	222	Tremors and clonic convulsions	None	(12)
B-85	Phenyl	*o*-Hydroxyphenyl	Methyl	14–443 (9)	222	111	Dorsiflexion of tail; tremors and violent convulsions; delayed death	None	(16)
B-77	Phenyl	*m*-Hydroxyphenyl	Methyl	18–886 (9)	443	222	Grogginess; tremors and mild convulsions	None	(16)
B-81	Phenyl	*p*-Hydroxyphenyl	Methyl	55–886 (5)	886	443	Tremors and clonic convulsions	None	(16)

B-90	Phenyl	*o*-Methoxyphenyl	Methyl	16–886 (9)	111	55	Tremors and violent convulsions	None	(16)
B-97	Phenyl	*m*-Methoxyphenyl	Methyl	16–886 (8)	443	222	Violent convulsions	None	(16)
B-89	Phenyl	*p*-Methoxyphenyl	Ethyl	55–886 (5)	222	111	Violent convulsions	None	(16)
B-87	Phenyl	*p*-Methoxyphenyl	Allyl	16–886 (8)	443	222	Tremors and violent convulsions	None	(16)
B-70	Phenyl	*p*-Methoxyphenyl	2-Hydroxyethyl	37–443 (7)	111	84	Tremors and violent convulsions	None	(16)
B-83	Phenyl	*m*-Ethoxyphenyl	Methyl	21–443 (8)	222	111	Dorsiflexion of tail; tremors and violent convulsions	Very slight	(16)
B-74	Phenyl	3-Methoxy-4-hydroxyphenyl	Methyl	28–672 (9)	672	443	Tremors and violent convulsions	None	(16)
B-82	Phenyl	3-Methoxy-2-hydroxyphenyl	Methyl	16–443 (8)	111	55	Tremors and clonic convulsions	None	(16)
B-80	Phenyl	3,4-Dimethoxyphenyl	Methyl	21–886 (9)	222	111	Clonic convulsions	None	(16)
B-75	Phenyl	2,3-Dimethoxyphenyl	Methyl	25–886 (9)	111	55	Dorsiflexion of tail; tremors and clonic convulsions	None	(16)
B-79	Phenyl	3,4-Methylenedioxyphenyl	Methyl	24–886 (8)	111	55	Tremors and violent convulsions; delayed death	None	(16)
B-76	Phenyl	*p*-Dimethylaminophenyl	Methyl	24–443 (8)	111	55	Dorsiflexion of tail; tremors and clonic convulsions	None	(16)
B-38	Phenyl	Benzyl	Hydrogen	14–443 (11)	55	28	Tremors and clonic convulsions	Very slight (?)	(17)
B-18	Phenyl	Benzyl	2-Hydroxyethyl	48–886 (9)	73	84	Violent convulsions	None	(12)



	Ar	Ar′	Y					
B-50	Phenyl	Phenyl	Amino	37–886 (13)	222	111	Tremors and violent convulsions	None	(18)
B-31	Phenyl	Phenyl	Diethylamino	55–886 (7)	886	443	Violent convulsions	None	(12)
B-41	Phenyl	Phenyl	Methylamino	18–443 (19)	222	111	Tremors and violent convulsions	None	(12)
B-19	Phenyl	Phenyl	1-Piperidyl	55–1344 (9)	222	886	Dorsiflexion of tail; tremors and violent convulsions	None	(12)
B-46	Phenyl	Phenyl	4-Morpholinyl	55–1554 (7)	1554	1170	Tremors and rolling movements; delayed death	None	(12)
B-45	Phenyl	*p*-Methoxyphenyl	Methylamino	48–886 (12)	443	222	Tremors and violent convulsions	Slight	(14)
B-49	Phenyl	Cyclohexyl	1-Piperidyl	55–1773 (12)	1773	1344	None given; 1773 "very sick"; delayed death	None	(12)
B-37	*p*-Methoxyphenyl	*p*-Methoxyphenyl	1-Piperidyl	55–1773 (10)	1773	886	Dorsiflexion of tail; tremors and clonic convulsions; intense salivation with 1773	Slight	(12)
B-67	*p*-Methoxyphenyl	Cyclohexyl	1-Piperidyl	55–1773 (7)	...	1773	Quiet and normal with 1773	None	(12)

(*Continued on p. 358*)

TABLE I.—FORMULAS, COMPARATIVE TOXICITY, AND ANALGETIC EFFECTIVENESS OF 67 ARYLALKAMINES (*Continued*)

Code No.	Ar	Ar′	Y	Dose Range, Mg./Kg. and No. of Mice ( )	Lowest Dose Fatal to Any Mouse	Highest Dose Survived by Any Mouse	Toxic Symptoms	Pain Depression	References
B-68	*p*-Methoxyphenyl	Cyclohexyl	Methylamino	28–443 (8)	443	222	Tremors and violent clonic convulsions	None	(12)
B-44	β-Naphthyl	Phenyl	1-Piperidyl	55–1773 (7)	...	1773	None recorded	None	(12)

	Ar	Ar′	R						
B-10	Phenyl	Phenyl	Hydrogen	55–886 (8)	84	111	Violent convulsions	None	(19)
B-13	Phenyl	Phenyl	Methyl	42–443 (10)	222	111	Violent clonic convulsions	None	(19)
B-14	Phenyl	Phenyl	Ethyl	32–886 (10)	55	55	Clonic convulsions	Slight	(14)
B-17	Phenyl	*p*-Chlorophenyl	Methyl	55–886 (9)	127	443	Dorsiflexion of tail; tremors and violent clonic convulsions	None	(12)
B-11	β-Naphthyl	Phenyl	Hydrogen	28–886 (9)	111	55	Tremors and clonic convulsions	Slight	(21)
	Miscellaneous Amines as Hydrochlorides								
B-54	N-Methyl-1,2-diphenylmethylamine. (N-Methyl benzohydrylamine)[c]			24–886 (12)	111	55	Clonic convulsions	None	(14)
B-55	N-Methyl-1-phenyl-1-cyclohexylmethylamine			24–886 (10)	42	55	Tremors and violent convulsions	None	(12)
B-69	1-Phenyl-1-Δ^1-cyclohexenylmethylamine			28–443 (9)	222	111	Tremors and violent convulsions	None	..
B-36	Ethyl α-(1-piperidyl)phenylacetate			55–4680 (9)	1773	1773	Clonic convulsions; delayed death	None	(12)
B-23	3,4-Dihydro-2-(1-piperidyl)-1-(2)-naphthalenone. (2-Piperidyl-1-tetralone)[c]			55–886 (8)	222	111	Death without convulsions	None	(23)
B-91	3-Phenyl-3-diethylaminoethylbenzofuranone-2. (Abbott's A. P. 43)[c]			16–886 (9)	886	443	Tremors and violent clonic convulsions	None	(24)
B-39	Ethyl α-allylaminophenylacetate			55–3547 (13)	3547	2037	Violent convulsions with 3547	None	(12)
B-4	1,2 Di-*p*-methoxyphenylbutyl amine			55–585 (10)	509	443	Tremors and violent clonic convulsions	None	(12)
	Morphine Sulfate			18–509 (47)	222	509	Dorsiflexion of tail	Very marked	
	Meperidine Hydrochloride			39–254 (32)	96	96	Dorsiflexion of tail; violent clonic convulsions	Distinct	
	Papaverine Hydrochloride			55–886 (11)	672	772	Tranquility; clonic convulsions	None	
	Antipyrine			400–700 (4)	700	600	Tremors and convulsions	None	
	Aminopyrine			100–400 (6)	400	400	Convulsions	Very slight	
	Sodium Salicylate			100–600 (5)	...	600	None recorded	None	

[a] Six had been given morphine sulfate ten days earlier. [b] Thirteen had been given morphine sulfate ten days earlier. [c] Synonyms.

TABLE II.—TYPICAL EXAMPLES OF ANALGETIC ACTIVITY AS TESTED BY THE HOT PLATE METHOD IN ALBINO MICE

Name of Drug	Dose, Mg./Kg.	Control	Reaction Time[a] Minutes after Injection 10	20	30	40	50	60	70	80	90	100	110	120
Morphine Sulfate	73	5	12	30	30	30	30	30	19	24	18	18	12	12
	35	3	24	30	30	30	30	27	21	19	15	15	12	12
Meperidine Hydrochloride	60	6	30	30	30	30	30	17	7	13	12	7	9	
	50	7	30	15	30	30	15	11	23	18	15	9		
	32	6	15	30	30	30	30	12	5	6	3	3		
B-7 Iso-1,2-diphenylethanolamine Hydrochloride	127	8	..	20	30	26	15	19	12	6	6			
	96	6	8	7	30	24	15	23	19	11	4			
	84	4	8	8	29	15	24	22	12	3	6			
B-45 N-methyl-1-*p*-methoxyphenyl-2-phenylethylamine Hydrochloride	55	6	6	12	15	15	15	12	10	4				
	48	6	4	12	12	30	15	15	12	5				

[a] The reaction time was taken in seconds every ten minutes. Unless the mice showed signs of pain they were removed from the hot plate after thirty seconds. The temperature of the plate was 60°.

the hot plate for thirty seconds and its standard error. In case of the more feebly acting substances, it would be necessary either to adopt a limit lower than thirty seconds, say twenty or fifteen seconds (see Table II), and then, of course, apply the same limit to morphine or whichever drug is used for comparison, to make the comparison with a specified suboptimal dose of morphine or meperidine or with a weaker analgetic than either of these, or to use a lower hot plate temperature, say 50°, instead of 60°; a combination of these modifications may also be employed.

SUMMARY

Of 67 arylalkamines compared with morphine and meperidine activity by the hot plate method in mice only iso-1,2-diphenylethanolamine hydrochloride produced a fair depression to pain. Slight action was demonstrable with β-morpholinoethyl-1,2-diphenylethylamine dihydrochloride, N-methyl-1,2-di-(*p*-anisyl)ethylamine hydrochloride, 1-*p*-methoxyphenyl-2-phenylethylmethylamine hydrochloride, α-N-piperidyldesoxyanisoin hydrochloride 1,1-diphenyl-2-aminobutanol hydrochloride, and 1-phenyl-1-β-naphthyl-2-aminoethanol hydrochloride.

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Notes

A Note on the Diet and Care of Cats in the Laboratory*

By JOHN F. FRICK and LLOYD C. MILLER†

THE care of domestic cats in the laboratory has presented difficult problems. Regardless of their previous environment, many cats react to confinement under laboratory conditions by refusing food. Unfortunately, this frequently reaches the point that the animal's life is endangered by actual inanition or by disease brought on by the self-imposed starvation. Therefore, it appears worthy of reporting on a ration which is relatively inexpensive and generally available commercially, for which cats show a spontaneous liking almost without exception. The ration consists of a mixture of Carno,[1] a product containing a high content of animal protein and fat, and Kasco,[2] a dry, commercial food for dogs. Carno, which is sold in cans ready for use, is semi-solid and apparently for this reason lacks somewhat in appeal to cats. The addition of enough Kasco to make a mixture stiff enough to hold its form in a food pan appears to increase its palatability a great deal. Cats have been maintained in this laboratory on a daily ration of 100 to 150 Gm. of this mixture for several months in perfect health and without any supplementation with fresh milk.

It has been noted that under this dietary regime, the excreta give off much less odor. With the cats housed in individual cages having floors of hardware cloth and pans beneath, the use of bagasse[3] in the pans for catching the excreta has materially simplified the problem of routine care. It is highly absorbent and may be disposed of easily. The entire cages are sterilized in steam once a week. The overall result is a significant reduction in the cost of care and an improvement in the cats' health and usefulness. The latter is an important advantage from the standpoint of their suitability for long-term experiments.

Because of the practical and humanitarian aspects involved, it seems appropriate to take this means of making known this favorable experience.

* Received April 20, 1950, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

† Present address: Director of Revision, United States Pharmacopeia, New York City.

[1] Carno—A preparation made from poultry by-products. Packed by C. A. Swanson and Son, Omaha, Nebr. We are indebted to Mark L. Morris, D.V.M., Raritan, N. J., for calling our attention to this product.

[2] Kasco Dog Ration—Kasco Mills, Inc., Waverly, N. Y.

[3] Staz-Dry is a commercially available form of bagasse obtainable at most stores carrying poultry and feed supplies.

Absence of Toxic Effects from Single Injections of Crystalline Vitamin B_{12}*

By CHARLES A. WINTER and CHARLES W. MUSHETT

THE acute toxicity of vitamin B_{12} is of importance, since the vitamin is now being used clinically with increasing frequency in amounts larger than has been found necessary for routine treatment of pernicious anemia. Extensive studies of the pharmacological properties including acute and chronic toxicity of crystalline vitamin B_{12} have been in progress in this laboratory for over a year. It was necessary to establish the nontoxicity of large doses of the crystalline material before it could be released to physicians for trial in conditions other than pernicious anemia.

In a recent report, Traina (1) described toxic reactions and deaths in mice treated with a concentrate containing vitamin B_{12}; intraperitoneal or subcutaneous injections of 3 mg./Kg. of B_{12} produced death in 100% of the treated animals. Since Traina's results on B_{12} concentrate are not in agreement with ours, using the crystalline substance, we are presenting some of our findings on acute administration at this time.

We have administered Crystalline Vitamin B_{12}, Merck, to mice in the following doses intraperitoneally: 100, 200, 400, and 1600 mg./Kg., and in doses of 800 and 1600 mg./Kg. intravenously. In addition, the material has been injected intraperitoneally in rats and guinea pigs in doses up to 100 mg./Kg. In no instances were there any deaths, or toxic manifestations. Some of the mice were sacrificed for pathological study forty-eight hours after injection, while others were observed for a week following treatment. Individual weight records were kept on the latter animals; the average values are given in Table I, which also shows the number of mice used. No gross pathological changes were observed in the animals at autopsy.

* Received April 15, 1950, from Merck Institute for Therapeutic Research, Rahway, N. J.

TABLE I.—ABSENCE OF TOXIC EFFECTS IN ALBINO MICE FROM SINGLE INJECTIONS OF LARGE DOSES OF CRYSTALLINE VITAMIN B_{12}

Sex	Dose, Mg./Kg.	Mortality Ratio, Dead/Total	Av. Body Weight, Gm. Initial	Av. Body Weight, Gm. 1 Week
M	100 i. p.	0/10	20.3	25.1
M	200 i. p.	0/10	20.5	24.8
M	400 i. p.	0/10	20.5	25.0
M	1600 i. p.	0/4	...	...
F	800 i. v.	0/3	...	...
F	1600 i. v.	0/3	...	...
M	Uninjected controls	0/15	21.0	24.8

Since the largest doses employed by us are more than 500 times as great as those of the B_{12} concentrate used by Traina, it appears that the toxic manifestations which he observed were probably due either to toxic impurities in the preparation which he used, or to contamination of the sample. It is clear from our results that crystalline vitamin B_{12} is essentially a nontoxic substance.

Results of the pharmacological study of vitamin B_{12}, including acute and chronic toxicity, will be published in detail at a later date.

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A Note Concerning the Incompatibility of Vitamin B_{12} and Ascorbic Acid*

By N. R. TRENNER, R. P. BUHS, F. A. BACHER, and W. C. GAKENHEIMER

IN A preliminary note, one of us reported some observations in connection with the incompatibility of vitamin B_{12}-ascorbic acid mixtures (1). Subsequent investigations of this compatibility problem have resulted in the discovery of a number of additional facts of considerable practical significance which we feel it important to report at this time.

The existence of a second biologically active, chemically distinct analog of vitamin B_{12} has been described by Kaczka, Wolf, and Folkers (2). This substance was designated by these investigators as vitamin B_{12a}. The existence of vitamin B_{12a} and other possible analogs appears to have considerable significance in connection with the compatibility of certain vitamin B_{12} concentrates and ascorbic acid.

We have now found that aqueous solutions of highly purified crystalline vitamin B_{12} preparations show a much higher degree of stability in the presence of pure ascorbic acid than previously reported. Thus aqueous solutions containing about 20 μg. of pure vitamin B_{12} per ml. and about 20 mg. of pure ascorbic acid per ml. were found to lose approximately 1.5% of their vitamin B_{12} content per day when stored at room temperature. Such solutions were found to have a *p*H between 2.5 and 3.0. Vitamin B_{12a}, on the other hand, when examined under the same conditions showed practically complete loss of color and activity in one day or less. Similar losses in one day or less were also observed when only 2 moles of ascorbic acid per mole of vitamin B_{12a} (concentration 20 μg. per ml.) were used. This reaction was observed to be the faster the higher the *p*H of the reaction system, it being complete in less than one hour at *p*H 7. At *p*H 0–1, on the other hand, the reaction between vitamin B_{12a} and ascorbic acid was observed to be very much slower than at *p*H 3. This *p*H dependence appears to suggest that the ascorbate ion rather than the undissociated acid is the other reactant. The addition of ascorbic acid to an aqueous solution containing equal amounts of vitamin B_{12} and vitamin B_{12a} resulted in the loss of the light absorption due to the vitamin B_{12a} in about twenty hours at room temperature followed by no further loss on additional standing for a period of several days, in complete accord with the behavior of the individual compounds.

Examination of a number of commercial vitamin B_{12} preparations for ascorbic acid compatibility revealed that they covered the whole range of compatibilities from complete loss of activity, through partial loss, to practically complete stability after forty-eight-hour test periods. In the light of our present findings, we are inclined to interpret such results as evidence of the presence, in varying amounts, of ascorbic acid-incompatible analogs of vitamin B_{12} in some commercial preparations.

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* Received April 8, 1950, from the Research Laboratories, Merck & Co., Inc., Rahway, N. J.

An Ink Writer for Recording Blood Pressure, Respiration, and Other Responses in Small Animals*

By THOMAS J. HALEY

THE usual methods for the kymographic recording of blood pressure, respiration, and other responses in small animals require the use of the smoked drum and fixation of the completed record. That these procedures are not entirely satisfactory is evident by the reports of ink writing and photographic methods in the literature. Marsh (2) has proposed the use of the strain-gauge manometer, but the expense involved has prevented its widespread use. Anderson (1) has described an ink writing mercury manometer, but it is useful with large animals only.

During the course of some screening experiments, the following pen was found to be useful for most types of pharmacological work. The plastic tubing used in hospitals for the intravenous administration of large volumes of fluid furnished the material required. The end of the tubing which is attached to the intravenous needle is composed of two pieces, the outer flexible plastic and an inner core of hard, lightweight plastic. This inner plastic tube is removed and cut as shown in Fig. 1. The upper lip *A* is used as the point of attachment to the lever arm. The bottom of the cut tube is closed with

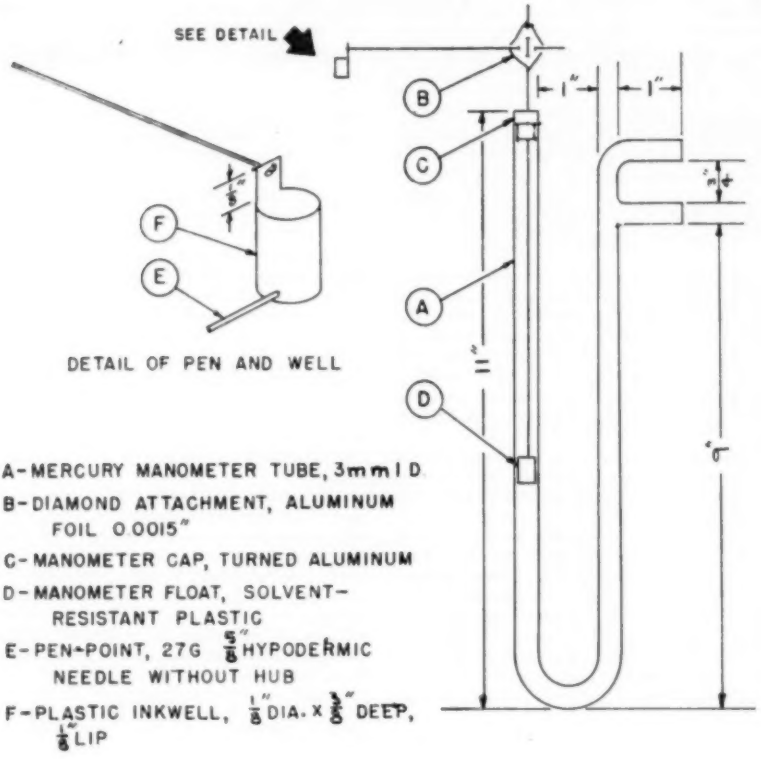

Fig. 1.—Assembly for ink writer.

model airplane cement, thus forming an ink reservoir 2/3 in. deep and 1/8 in. in diameter. The pen point is made by cutting the hub from a 27 G., 5/8-in. hypodermic needle and forcing this needle, beveled edge up, through the plastic ink reservoir

* Received December 28, 1949, from the School of Medicine, University of California at Los Angeles.
This paper is based on work performed under Contract No. AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

just above the cement bottom. The writing point should be perpendicular to the lever arm. The pen is attached to the lever arm by forcing the lever arm through a small hole in the attachment lip. If necessary, a small drop of cement may be placed at this point. Figure 1, besides showing a detailed drawing of the pen, also illustrates the construction of a manometer which can be used as a mercury manometer for recording blood pressure or as a chloroform manometer for recording intestinal movements. When the manometer is used to record blood pressure in the rat or guinea pig, the animal should be given 0.1 cc. of heparin solution (1000 Toronto Units) intravenously and connected to the manometer with tubing filled with physiological saline solution. Figure 2 illustrates the results which can be obtained with such a system. At times, because of the inertia of a mercury manometer, it is desirable to use a membrane manometer such as has been described by D'Amour and Blood (3). In such cases, the pen is attached in the manner illustrated in Fig. 1. Also, the pen may be attached to heart levers and tambour lever arms for recording a variety of physiological responses.

When the pen has been attached to the lever arm, the ink reservoir is filled with commercial ink (Phipps-Bird) using an eye dropper with a very fine tip, the hypodermic needle stylet inserted in the pen point and moved back and forth to start the ink flowing. The pen point is brought up to the paper and responses such as those shown in Fig. 2 recorded. When the record is complete the stylet is again inserted in the pen point, thus making it unnecessary to wash out the reservoir and thoroughly clean it each time after use. We have employed such pens for over one year and always obtain excellent results. Because it is inexpensive and because most laboratories can adapt the pen to fit existing equipment, we believe the pen will be very useful in the teaching of experimental physiology and pharmacology.

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Book Reviews

Federal Food, Drug, and Cosmetic Act. Judicial and Administrative Record, 1938–1949. By VINCENT A. KLEINFELD and CHARLES WESLEY DUNN. Commerce Clearing House, Inc., New York, 1949. xxvi + 895 pp. 16.5 x 25 cm. Price $17.50.

This valuable book will fill a need for a comprehensive record of the judicial and administrative actions under the Federal Food, Drug, and Cosmetic Act covering the period since its enactment in 1938 to May, 1949. It is a reference book so logically arranged and so clearly written that it can be used advantageously by anyone whose business or profession is regulated by the Act. The professional practice of every pharmacist who sells or dispenses drugs which at some time in the past crossed a state line is subject to such regulation. From a study of *Federal Food, Drug, and Cosmetic Act 1938–1949* it is possible to acquire a practical working knowledge of the Act as it affects many pharmaceutical transactions and activities. By the intelligent use of the book as a reference, it is possible to find an analogy to almost any question involving the Food, Drug, and Cosmetic Act that might arise.

The book is divided into three basic sections. The first part contains a digest of every opinion of record rendered under the Act. References to the subsections of the Act involved in each instance are cited.

For a number of years the Food and Drug Administration made available the essential parts of informal answers to questions and statements of policy involving interpretations of general interest of many provisions of the Act. In the second portion of the book by Dunn and Kleinfeld under the section entitled: "Trade Correspondence" all of these informal announcements and answers by the Administration are reproduced. By reference to this section many administrative interpretations of the Act may be found.

The third section includes a useful table of references from the law to interpretations of it, together with easily understandable directions for its use.

The book seems to have been designed throughout for ready reference and use either by lawyers or by laymen. A feature which contributes to this design is an unusually exhaustive and comprehensive index. The typography is well chosen and the book is legibly printed.

Multi-Enzyme Systems. By MALCOLM DIXON. Cambridge University Press, New York, 1949. 100 pp. 12.5 x 19 cm. Price $1.75.

This book is composed of four lectures on enzymes presented by the author at Cambridge University, these having been recorded, presumably, at the time of lecturing, for the charm of the lecturer is well preserved. The clarity and simplicity with which the material is presented will fascinate scientific lecturers in any field. The author builds up complex multi-enzyme systems by beginning with simple

theoretical examples and classifications, and within a few short pages he progresses without stumbling to the complete carbohydrate cycle. He does not go into sufficient detail to make the book of great value as reference material, but his approach to the formation of a logical and clear picture of enzymic systems will probably be refreshing even to the most inveterate enzyme chemist. For example, he describes the peculiar position of certain coenzymes as "the remarkable state of affairs, peculiar to living matter, of a system of unstable catalysts being kept in existence by the occurrence of the reactions which they catalyse." Illustrating the equilibrium state involved in multi-enzyme systems he says, "An object placed on an inclined endless belt, on which it slides. . . can only be kept in the same position by continuously winding up the belt, thereby expending energy, which is continuously converted into heat. . . This kind of state is especially characteristic of living matter."

This book may bring new ideas to minds of enzyme researchers by its brief references to the significance to the organism of such phenomena as the close juxtaposition of related enzymes within the cell, and the possibility of the use of highly unstable substrate molecules. The author discusses the scale of hydrogen potential and the convenience of its use due to the direct relationship between rH and free energy, each rH unit being equal to 1380 calories at 30°. Several rules for the application of rH data are given, and the author applies these rules to many oxidative systems. Particularly interesting is his application of the rH system to phosphorylases.

Introductory Botany. By ALEXANDER NELSON. The Chronica Botanica Company, Waltham, Mass., 1949. viii + 479 pp. Illustrated. 14 x 22 cm. Price $3.75.

The title of this textbook has considerable meaning for the book does treat the several phases of botany in a real introductory fashion. The author has attempted not to overemphasize any one subject of botany to the jeopardy of another. Thus, he has divided the text into three major sections giving about equal distribution to each.

The text is not intended to lead students into a specialization of any one phase of botany. In fact the author has indicated that a second volume will follow and this will seek to specialize in the medical aspects of botany. As the text is compiled it is definitely more valuable for teaching purposes than a specialized botany would be because it contains the fundamentals upon which any applied field of botany should be developed. In this respect it is well suited for the pharmacy student. It is true that some teachers might seek to supplement the introductory material by using drug plants as examples whenever possible. More data relative to plant chemistry might have also been included. These are not necessary adjuvants to the beginning course, however, and where the text is deficient in this respect it more than fills the bill with its general coverage of basic principles of botany.

Two outstanding features of the text are its excellent introductory treatment of plant physiology, an important phase for modern courses in pharmacognosy, and the many diagrammatic illustrations. The latter should be most helpful for teaching purposes.—H. W. YOUNGKEN, JR.

Selenium. By SAM F. TRELEASE and ORVILLE A. BEATH. Published by the authors, New York, 1949. x + 292 pp. Illustrated. 11 x 18.6 cm. Price $5.50.

This important monograph by two leading authorities on biological and biochemical aspects of selenium research will be of considerable interest to progressive pharmacists. Although symptoms of selenium poisoning were recognized in the United States nearly one hundred years ago, it was only about twenty years ago that the cause of the symptoms was definitely proved and the magnitude of the problem was realized. In the last two decades selenium has become a major concern of public health officers, agriculturalists, and ranches, especially in the western United States. It is because of the tremendous implications of this element in public health, a subject ably and intelligently discussed by the authors, that pharmacists will find major interest in this book.

Selenium is unique among the elements in that several important species of plants can, without themselves being injured by it, absorb and accumulate selenium from seleniferous soils in quantities that render the plants toxic (and often lethal) to animals including man. The implications of this phenomenon are tremendous not only for the livestock industries but for agriculture and the public in general. To realize this one need only consider the potentially tragic consequences if an unusually high percentage of seleniferous wheat should find its way into milling establishments and be turned out as flour for use of bakers and housewives.

The authors tell their story in an interesting way in a most logical sequence consisting of (I) history, (II) selenium indicator plants, (III) geology, (IV) toxic forages and crops, (V) selenium poisoning in animals and (VI) in man. Control measures are suggested in Chapter VII, and Chapter VIII concerns the chemistry of selenium. The book is profusely and very well illustrated with photographs, diagrams, and graphs.

Professors Trelease and Beath have been among the foremost investigators of the selenium problem for nearly two decades and, therefore, write with authority. They also write with a clarity that makes for interesting as well as informative reading.—ROBERTSON PRATT.

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